Targeting Vascular Tissue Factor Expression:

Pathophysiology and Clinical Implications

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Certification of originality

I herewith certify that all study designs, concepts and data presented in this thesis result from no other sources than my personal work, unless stated otherwise. This work was conducted in the facility of the Cardiovascular Research at the Institute of Physiology, University of Zurich between September 2006 and April 2011. The work related to this thesis was published by myself as a first author in the following articles:

Dietary Alpha-Linolenic Acid Inhibits Arterial Thrombus Formation, Tissue Factor Expression, and Platelet Activation. Holy EW, Forestier M, Richter EK, Akhmedov A, Leiber L, Camici GG, Mocharla P, Lüscher TF, Beer JH, Tanner FC. *Arterioscl Thromb Vasc Biol*. 2011; 31: 1772-80.

Tissue factor in cardiovascular disease: pathophysiology and pharmacological intervention. Holy EW, Tanner FC. *Adv Pharmacol*. 2010;59:259-92.

Laminin receptor activation inhibits endothelial tissue factor expression. Holy EW, Stämpfli SF, Akhmedov A, Holm N, Camici GG, Lüscher TF, Tanner FC. *J Mol Cell Cardiol*. 2010;48:1138-45.

Berberine, a natural lipid-lowering drug, exerts prothrombotic effects on vascular cells. Holy EW, Akhmedov A, Lüscher TF, Tanner FC. *J Mol Cell Cardiol*. 2009;46:234-40.

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I. Summary

Arterial thrombosis is the critical event in acute vascular syndromes such as unstable angina pectoris, myocardial infarction, peripheral ischemia, and stroke. The fate of a forming thrombus is determined by the balance between pro-thrombotic mediators such as tissue factor (TF) and plasminogen activator inhibitor type 1 (PAI-1), and anti-thrombotic factors like tissue factor pathway inhibitor (TFPI) or tissue plasminogen activator (tPA). TF, a 263-residue membrane-bound glycoprotein, is a key protein for the activation of coagulation; it binds activated factor VII (FVIIa), which in turn activates factor X, ultimately leading to thrombin formation. TF expression is upregulated by several mediators such as tumor necrosis factor-α (TNF-α) or thrombin and can be detected in a variety of cell types in atheromatous plaques. In addition to the classical antithrombotic agents, drugs targeting TF and the TF:FVIIa complex have recently been introduced, since inhibiting the initiation phase may prevent thrombus formation more efficiently. Moreover, considering the non-hemostatic properties of the TF:VIIa complex, inhibition of TF may also interfere with the promigratory and proproliferative effects of TF.

In the present study we characterized the effect of compounds, which have been proposed to exert cardioprotective properties on the expression of prothrombotic markers in the vessel wall, in particular TF expression, by the use of cellular and in vivo models.

In the first project, we demonstrated that alpha-linolenic acid (ALA), a plant-derived n-3 fatty acid, potently inhibits TF expression and activity in vascular smooth muscle and endothelial cells. This effect was mediated at the transcriptional level by inhibition of the mitogen activated protein-kinase pathways and the transcription nuclear factor kappa-light-chain-enhancer of activated B-cells (NFkappaB or NFkB). In line with this observation mice fed an ALA-rich diet for 2 weeks suffered a

significantly delayed thrombotic occlusion following photochemical vessel wall injury as compared to controls. Moreover we could demonstrate that this antithrombotic effect was partially mediated by an inhibitory effect of ALA on platelet activation and aggregation. Hence, ALA may represent an attractive nutritional intervention with direct dual anti-thrombotic effects.

Epigallocatechin-3-gallate (EGCG) is a polyphenol found in green tea and experimental as well epidemiological studies revealed important cardioprotective properties of ECGC. Recently EGCG has been characterized as a ligand for the 67 kDa laminin receptor (67LR). In the second project we demonstrated that activation of the 67LR by laminin or EGCG inhibits cytokine induced TF expression in endothelial cells via the c-Jun N-terminal kinases 1 and 2, and identified 67LR as a potential target for the development of novel anti-thrombotic therapies.

Aim of the third project was to investigate whether the natural lipid lowering drug berberine (BBR), similar to statins, exerts pleiotropic effects on endothelial tissue factor (TF) expression. We showed that BBR, at physiologically relevant concentrations, enhances TF expression and in parallel decreases TFPI expression both in human endothelial cells and in arteries of $ApoE^{-/-}$ mice. Analysis of the mechanisms demonstrated that BBR enhanced cytokine induced TF expression through a posttranscriptional mechanism involving stabilization of TF mRNA. BBR may therefore favor the development of arterial thrombosis, in particular in patients with atherosclerotic lesions since inflammatory environments are often encountered under these conditions, and its use in clinical practice should be considered with caution until large-scale clinical trials have evaluated its safety.

Finally, in the last project we characterized the role of the phosphatidylinositol-3 kinase (PI3K) signaling pathway in the vasculature. The PI3K controls major cellular processes such as cell cycle, proliferation and differentiation. In the vasculature,

p110α is the predominant PI3k isoform expressed in endothelial and vascular smooth muscle cells. This study was designed to address the role of p110α in endothelial cell and vascular smooth muscle cell proliferation, migration, and activation, which are critically involved in the pathogenesis of atherothrombosis. We demonstrated that inhibition of p110α differentially regulates smooth muscle and endothelial cells. In addition to its potent antiproliferative and antimigratory effects on smooth muscle cells, targeting p110α also inhibits the expression of prothrombotic markers on endothelial such as TF and PAI-1. Considering that specific targeting of p110 isoforms is currently evaluated in different areas of modern medicine, we propose local p110α inhibition as a novel antithrombotic strategy in particular in the context of drug eluting stent design, in order to prevent restenosis without adding the risk of enhanced thrombogenicity.

In summary this study supports the concept that modulation of TF expression in the vasculature represents an attractive target in preventing arterial thrombus formation associated with the development of cardiovascular events and proposes new strategies to inhibit TF expression. Hence, this work may contribute to the development of new potent antithrombotic drugs.

II. Zusammenfassung

Die arterielle Thromboseentstehung ist der kritische Schritt in der Entstehung akuter vaskulärer Ereignisse. Die Entwicklung der arteriellen Thrombose ist durch das Gleichgewicht zwischen prothrombotischen Faktoren, wie zum Beispiel Gewebethromboplastin (tissue factor, TF) oder PAI-1 (plasminogen activator inhibitor type 1) und antithrombotischer Gegenspieler wie TFPI (tissue factor pathway inhibitor) oder tPA (tissue plasminogen activator) bestimmt. TF ist ein wichtiger Aktivator der Gerinnungskaskade; es bindet den aktivierten Faktor VII (FVIIa), welcher im Gegenzug Faktor X (FX) aktiviert und somit die Thrombin Bildung einleitet. TF wird innerhalb der atherosklerotischen Plaque in verschiedenen Zelltypen synthetisiert und dessen Expression durch etliche Entzündungs Mediatoren gesteigert. Da eine Hemmung der Initiierung der Gerinnungskaskade möglicherweise eine potentere antithrombotische Wirkung auf die Entstehung der arteriellen Thrombose ausübt erscheint die gezielte Hemmung der Aktivität von TF und des TF:FVIIa Komplexes eine neue attraktive Strategie darzustellen.

In der vorliegenden Studie wurden Substanzen, welche bisher aufgrund ihrer antiatherogenen Eigenschaften als kardioprotektiv gelten, auf ihr antithrombotisches Potential, insbesondere in Bezug auf TF Expression, charakterisiert.

Im Rahmen des ersten Projekts konnten wir zeigen, dass die pflanzliche n-3 Fettsäure alpha-Linolen Säure (ALA) TF sowohl in Gefässmuskel als auch Endothelzellen auf transkriptioneller Ebene hemmt. Diese Hemmung beruht auf die verminderte Aktivität MAP kinasen sowie des Transkriptionsfaktor NFkappaB. Desweiteren konnten wir nachweisen, dass ALA die Aktivierung von Plättchen und deren Aggregation signifikant hemmt. Entsprechend dieser Beobachtungen führt eine ALA-reiche Diät zu einer verzögerten Thromboseentstehung in einem entsprechenden Mausmodell der arteriellen Thrombose.

Sowohl experimentelle als auch epidemiologische Studien belegen, die kardioprotektiven und antiatherosklerotsichen Effekte des Grüntee Polyphenols Epigallocatechin-3-gallate (EGCG). Kürzlich konnte der 67 kDa Laminin Rezeptor (67LR) als zellulärer Rezeptor für EGCG charakterisiert werden. In dem zweiten Projekt dieser Studie konnten wir nachweisen, dass die Aktivierung von 67LR durch EGCG oder die extrazelluläre Matrixkomponente Laminin die Zytokin induzierte Expression und Aktivität von TF in Endothelzellen über die c-Jun N-terminal Kinasen 1 und 2 hemmt. Somit erscheint die Aktivierung von 67LR eine neuartige antithrombotische Therapieoption.

Ansatz des dritten Projektes war es nachzuweisen ob der natürliche Lipidsenker Berberine (BBR) ähnliche pleiotrope antithrombotische Effekte auf TF besitzt wie die Wirkstoffe aus Stratingruppe. Wir konnte zeigen, dass BBR in physiologisch relevanten Konzentrationen, sowohl in den Karotiden behandelter Mäuse, als auch in Endothelzellen in vitro, TF überexprimiert und im Gegenzug die Expression von TFPI hemmt. Somit führt BBR zu einem signifikanten Anstieg der TF Aktivität in vivo und in vitro. Dieser Effekt beruht auf eine eine posttranskriptionelle Stabilisierung der TF mRNA. BBR begünstigt somit im Gegensatz zu den klinisch etablierten Statinen ein prothrombotisches Umfeld in der Gefässwand. Diese Beobachtung ist von Relevanz insbesondere in Patienten mit vorbestehenden entzündlich veränderten atherosklerotischen Plaques in denen erhöhten Zytokinspiegel nachgeweisen werden können. Eine klinische Anwendung von BBR als Alternative oder in Kombination mit Statinen sollte daher, bei derzeit ausstehender Datenlage in Bezug auf den Einfluss von BBR auf die Rate kardiovaskulärer Ereignisse, nur mit Vorsicht erwogen werden.

Thema des letzten Projekts war es die Rolle der Phosphatidylinositol-3 kinase (PI3K) auf die Aktivierung von Gefässzellen zu studieren. Die PI3K reguliert

essentiele zelluläre Prozesse wie Zellzyklus, Proliferation und Zelldifferenzierung. In der Gefässwand stellt p110α die vorwiegende Isoform der PI3K dar, und ist sowohl in Endothel als auch Gefässmuskelzellen nachgewiesen worden. Ziel dieser Studie war es den Einfluss von p110α auf die Proliferation, Migrationsfähigkeit sowie Aktivierung in Endothel und Gefässmuskelzellen zu charakterisieren, welche in der Pathogenese der Atherothrombose eine kritische Rolle spielen. Wir konnten nachweisen, dass die Hemmung von p110α Muskel- und Endothelzellen unterschiedlich reguliert. Zusätzlich zu dem augeprägten antiproliferativen und antimigratorischen Effekt auf Gefässmuskelzellen, führt eine Hemmung der p110α Isoform zu einer Hemmung der TF und PAI-1 Expression in zytokinaktivierten Endothelzellen ohne deren Proliferation oder Migrationskapazität zu beeinträchtigen. Aufgrund dieser Ergebnisse stellt die lokale Hemmung der p110α Isoform in der Gefässwand somit eine neue therapeutische Optionen in der Entwicklung von medikamenten-beschichteten Stents dar, mit dem Ziel der Verhinderung einer lokalen Restenose und gleichzeitiger Reduzierung der Thrombogenität des Stents.

Zusammenfassend bestätigen die vorgestellten Ergebnisse, dass die Modulierung der TF Expression in der Gefässwand eine attraktive therapeutische Strategie zu Verhinderung der Entstehung arterieller Thrombosen darstellt, welche klinisch mit der Entwicklung kardiovaskuläre Ereignisse einhergeht.

III. Tissue factor: structure and functions

1.Tissue factor in coagulation

In intact vessels blood circulates as a fluid due to a physiological balance between natural procoagulant and anticoagulant factors. The clotting cascade defines a complex system of zymogens and enzymatic reactions designed to limit blood loss and ultimately restore vascular integrity by formation of a clot following vessel injury. Initially initiation of coagulation was thought to be triggered by two distinct pathways: the intrinsic, or contact activation, pathway and the extrinsic, or tissue factor, (TF) pathway. Even though the two pathways are independently activated, the distinction between both of them is blurred since they share a final common pathway and the discovery of cross talks between the extrinsic and intrinsic system. The intrinsic pathway is activated when blood or plasma comes in contact with subendothelial connective tissues or negatively charged surfaces and involves circulating factors such as factor XII, factor XI, factor IX, prekallikrein, and high molecular weight kininogen [1]. However, since congenital deficiency in contact factors, for example factor XII, does not result in an increased bleeding tendency or severe pathologies in humans, the role of the contact pathway in the maintenance of normal hemostasis has been questioned.

Coagulation through the TF pathway is activated when blood interacts with TF expressing cell surfaces after vascular injury or plaque rupture (figure 1). The initial step consists in the reversible binding of the zymogen factor VII (FVII) or the activated FVII (FVIIa) to membrane-bound TF [2,3]. This results in the formation of both TF:FVII and TF:VIIa complexes. However, only the latter exerts the enzymatic activity required for further activation of downstream proteases. Once bound to TF, the inactive FVII is converted to FVIIa via limited proteolysis by several plasma proteases, such as XIIa, Xa, IXa or thrombin generating a positive feedback loop[4].

The resulting TF:FVIIa complex is a potent activator of the coagulation cascade and is composed of a regulatory subunit, TF, which acts as a cofactor and the serine protease VIIa exerting catalytic functions [5]. The TF:VIIa complex activates factor IX to factor IXa which in association with VIIIa catalyzes the conversion of factor X to Xa. In addition, factor X is directly converted to factor Xa by the TF:FVIIa complex. Factor Xa in association with factor Va and divalent calcium form the so called prothrombinase complex which cleaves prothrombin to form thrombin [6]. Thrombin exerts direct effects on coagulation through conversion of fibrinogen to fibrin, which assembles to clots, activation of platelets and activation of various proteases of the coagulation cascade such as FVII, FXI, and FV in an auto-feedback loop. Beside its well-known role in clot formation functions of thrombin extend from coagulation to vascular remodeling processes such as proliferation of vascular smooth muscle and endothelial cell or stimulation of growth factor secretion, ultimately leading to restoration of vascular integrity [7-10].

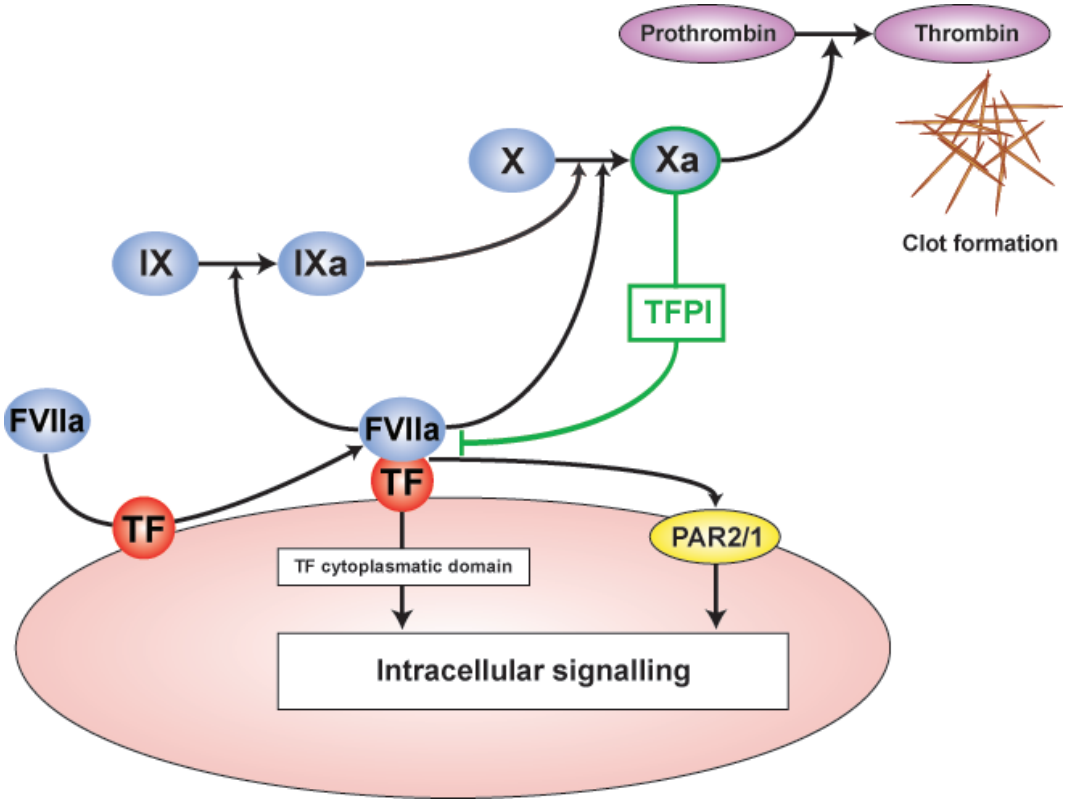

Figure 1: TF exerts extracellular effects as main trigger of coagulation as well as intracellular effects on signal transduction.
Binding of FVIIa to TF leads to the activation of FIX and FX. This results in thrombin generation and clot formation. The erndogenous inhibitor TFPI, binds to FXa thereby suppressing TF:FVIIa activity. Beside induction of the coagulation cascade, formation of the TF:FVIIa complex activates intracellular signaling pathways involved in the pathogenesis of cardiovascular disease. Holy et al. *Adv Pharmacol,* 2010;59:259-92.

2. Tissue factor protein structure

The TF (thromboplastin, CD142) gene located on chromosome 1 at p21-22 spans a 12.4 kb region and contains six exons encoding a single transmembrane polypeptide chain composed of 263 amino acids [11-14]. The TF protein is divided into three domains: an extracellular (219-amino acid), a membrane-spanning (23 amino acids) and a cytoplasmic (21 amino acids) region [6]. TF is considered a class I integral membrane protein since the amino-terminus of the protein is located extracellularly while the cytoplasmic tail contains the carboxy-terminus. Mobility studies on sodium dodecyl sulfate gels reveal that the molecular weight of the fully glycosylated protein is about 45’000 kDa [15]. The extracellular domain provides two high affinity sites for the binding of factor VII to TF. This binding is crucial for the induction of the conformal changes required to induce the catalytic activity of factor VII[16, 17]. In addition, anchoring of the TF protein to the membrane via the hydrophobic transmembrane domain has been demonstrated to be essential for procoagulant activity of the TF protein. However the nature of the anchoring domain does not seem to play a role[18]. Since deletion of the cytoplasmic tail is not paralleled by an impaired TF procoagulant activity [18], recent studies focused on possible non-hemostatic functions of this domain. Indeed, experiments performed mainly in fibroblasts and carcinoma cell lines revealed that phosphorylation of the cytoplasmic tail activates intracellular signaling pathways and induces cellular responses which are described in the following chapter.

In 2003 Bogdanov et al. characterized an alternatively spliced (as) form of TF mRNA lacking the fifth exon which encodes the transmembrane domain [19]. The translated

TF protein therefore lacks membrane anchorage and is detectable in the plasma as a soluble TF protein. The asTF protein has been described in several cell types and up to now its contribution to the TF procoagulant activity in the plasma as well as thrombus formation in vivo remains barely understood [20, 21].

3. Tissue factor: a signaling receptor

The tight link between hemostasis and progression of diseases such as atherosclerosis or inflammation provides the evidence for complex interactions between the coagulation cascade and pathophysiological processes. In particular because of its similarity to class II cytokine receptors recent studies suggested a non-hemostatic role of TF in several biolodical processes through induction of intracellular signaling events [22]. The following chapter provides an overview of TF mediated cell signaling.

Evidence for TF:VIIa induced signaling is derived from experiments in which FVIIa binding to TF was shown to induce intracellular calcium transients in different types of TF expressing cells [23, 24]. This observation was followed by studies demonstrating that binding of factor VIIa to TF induces the activation of the three major mitogen activated protein (MAP) kinase family members p42/p44 MAP kinase, p38 MAP kinase and c-Jun N-terminal kinase (JNK) [25, 26]. In fibroblasts the TF-FVIIa induced activation of Src-like kinases and the PI3-kinase leads to activation of the downstream Rho-like guanosine triphosphatases Rac and Cdc42, leading to cytoskeletal reorganization [27]. Interestingly, the induction of kinase activation was demonstrated to be dependent on the catalytic activity of factor VIIa because inactivated factor VII or anti-TF antibodies blunt these effects [23, 25, 26, 28].

By activating several kinases the TF:VIIa complex regulates the transcription of numerous genes involved in physiological and pathophysiological processes such as

cell migration, cell growth, or apoptosis. Indeed, studies demonstrated that binding of factor VIIa to TF induces activation of key transcription factors such as Egr-1 or nuclear factor kappa B and the activation of the RNA polymerase A [26, 29]. Microarray analysis of the gene product profiles further revealed increased mRNA levels of growth factors (i.e. connective tissue growth factors CCN1 and CCN2, fibroblast growth factor 5) and inflammatory cytokines (i.e. interleukin-1β, interleukin-8) following factor VIIa and TF interaction [28, 30].

The molecular mechanism by whitch the TF:VIIa complex transduces intracellular signaling involves G-protein-coupled, protease-activated receptors (PARs) [31, 32]. Currently 4 different PARs have been characterized: PAR1, PAR2, PAR3, PAR4. There is a growing evidence that TF:VIIa signaling is mediated mainly by PAR2 and to a lesser extend by PAR1, while PAR3 and PAR4 do not seem to be involved. For instance in fibroblasts derived from PAR1 knockout mice factor VIIa induced response only occurred when TF and PAR2 where co-expressed [30]. Moreover, data obtained in a carcinoma cell line demonstrate that antibodies targeting PAR2 but not PAR1 blunt TF:VIIa induced IL-8 expression and smooth muscle cell migration [33].

Even though not essential for TF:VIIa induced activation of MAP kinases and gene expression the cytoplasmic domain of TF is implicated in cytoskeletal organization and cell migration [34, 35]. Protein kinase C mediated phosphorylation of the cytoplasmic domain at the serine residues Ser253 and Ser258 enables the interaction of the cytoplasmic tail with the actin-binding protein 280 thereby regulating actin filament rearrangement [36, 37]. Hence, studies performed in mice lacking the cytoplasmic tail of TF demonstrated the crucial role of the cytoplasmic domain in vascular smooth muscle migration and vascular remodeling after injury [38].

Since increasing evidence suggests an essential role of TF as a key signal transducer beyond is well-known functions in coagulation, developing specific inhibitors that suppress either TF:VIIa mediated signaling or its coagulant function may offer interesting therapeutic approaches in the treatment of TF associated diseases.

IV. Tissue factor: implications in vascular disease

1. Tissue factor distribution

a) Tissue Factor in the vessel wall

Under normal hemostatic conditions TF is only barely expressed in endothelial cells (figure 2). Inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) [39, 40], interleukin-1β [41], and mediators including CD40 ligand [4], enhance TF expression *in vitro*. This induction of TF expression is mainly mediated by the mitogen-activated protein (MAP) kinases p38, extracellular-signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) which activate transcription factors such as nuclear factor kappa B, activator protein-1 or early growth-response gene product-1, all of them involved in the transcription of the TF gene [42-44]. Depending on the stimulus, induction of TF expression occurs via activation of either all three MAP kinases [39, 40, 45] or only a single pathway [44]. Protein kinase C as well as the Rho-kinase pathway have also been shown to mediate the induction of endothelial TF expression [44, 46, 47]. Whereas the MAP kinase, Rho-kinase and protein kinase C pathways regulate TF expression in a positive manner, the phosphoinositide 3-kinase pathway exerts a negative regulation on TF expression [48, 49]. Indeed, downstream targets of the phosphoinositide 3-kinase are able to regulate TF expression at both the transcriptional and the translational level. Akt and glycogen synthase kinase-3β regulate TF transcription [50], whereas the mammalian target of rapamycin and

p70S6 kinase inhibit TF translation [49, 51]. An additional mechanism affecting the regulation of TF protein expression is stabilization of TF messenger RNA. A recent study in endothelial cells demonstrated that a decrease in TF promoter activity may be counteracted by an increased TF mRNA stability leading to enhanced TF protein expression [39].

In contrast to endothelial cells vascular smooth muscle cells constitutively express TF and provide an hemostatic barrier after vascular injury [52-55]. Similar to endothelial cells, TF expression in vascular smooth muscle cells is enhanced by several receptor ligands such as CD40 ligand, histamine, thrombin, bacterial endotoxin, oxidized LDL, or C-reactive protein [55-59]. Even though not investigated to the same extend as in endothelial cells, the MAP kinase and phosphoinositide 3-kinase pathways regulate TF expression also in vascular smooth muscle cells.

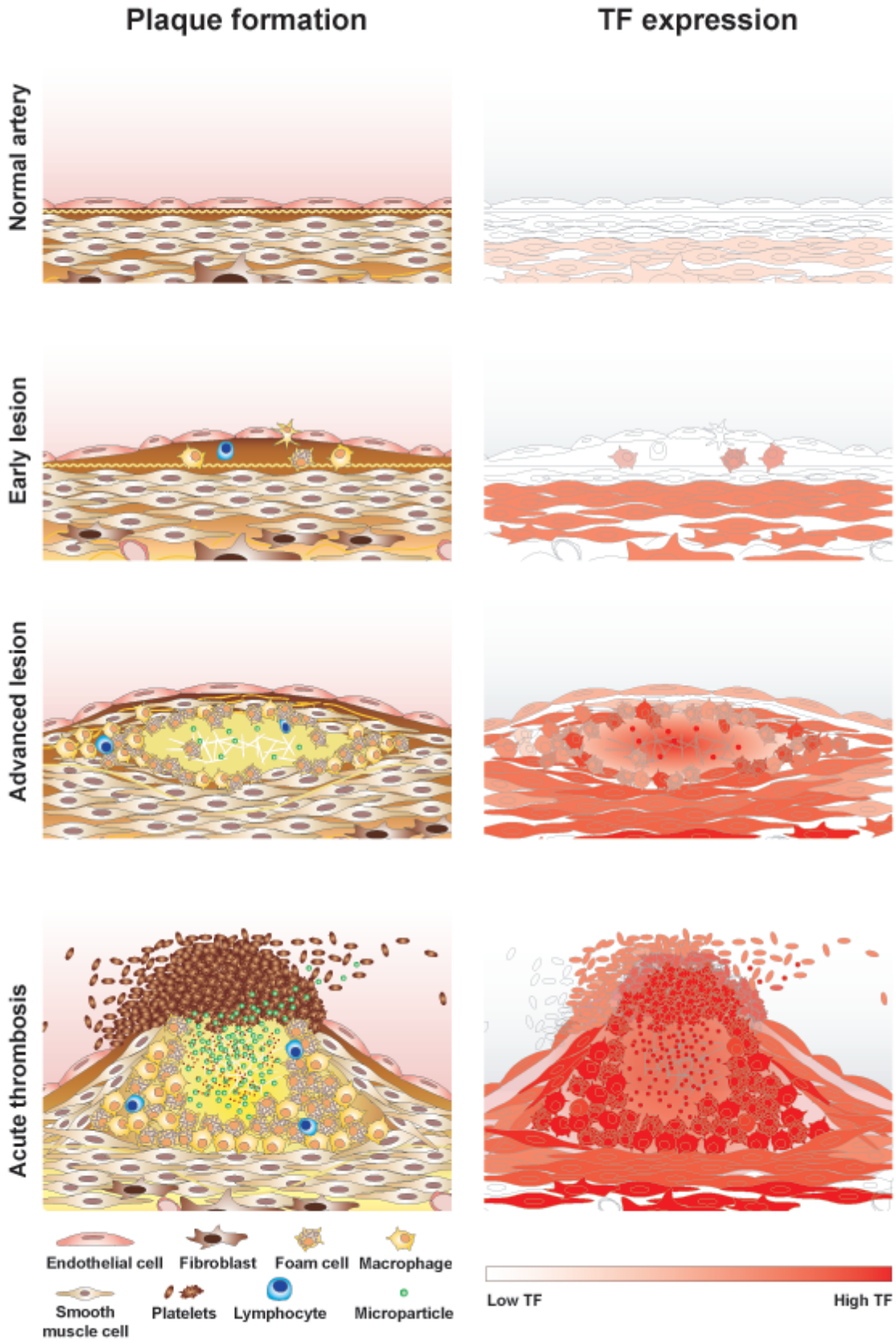

Figure 2: Expression of TF in different cell types during the pathogenesis of atherosclerotic lesions.
During plaque progression, the inflammatory environment triggers TF expression in endothelial cells, vascular smooth muscle cells, and macrophages/foam cells. On plaque rupture, the procoagulant material including TF-containing microparticles located in the necrotic core is released into the blood, leading to rapid initiation of coagulation, thrombus formation, and vessel occlusion. Holy et al. *Adv Pharmacol,* 2010;59:259-92.

b. Blood-borne tissue factor

More recently TF has been also been detected at low levels in the plasma and urine and these levels increase in several disease conditions such as atherosclerosis, disseminated intravascular coagulation, glomerulonephritis, sepsis, or diabetes.

Monocytes are thought to contribute as a major source to the plasmatic TF pool. Monocytes constitutively express TF and its expression is enhanced after exposure to numerous inflammatory mediators [60-65]. Indeed, elevated levels of TF have been measured in the plasma during septic states and may account for the thrombotic complications encountered in those patients [66, 67]. Endotoxin-induced TF expression is mediated via MAP kinases [63, 64] and the consecutive activation of the transcription factors EGR-1, c-Fos/c-Jun and nuclear factor kappa B. In addition endotoxin also increases TF mRNA stability [68].

TF has also been described in circulating granulocytes and eosinophiles as well as neutrophils and basophils have been found to express TF [69-73]. TF translocation to the cell surface as well as transcription is enhanced upon stimulation of those cells with granulocyte/macrophage colony-stimulating factor or platelet-activating factor [69]. These findings may explain the pro-thrombotic state associated with hypereosinophilia [74].

Even though TF-containing platelets have been described by several groups [70, 75-78] the role of platelet derived TF remains debatable [79]. In platelets TF is encountered in different compartments including the membrane the matrix of α-granules as well as canalicular system [63], and translocated to the cell surface upon activation by different agonists [63]. Since TF messenger RNA is not found in human megakaryocytes the source of platelet derived TF remains a matter of debate. One hypothesis suggests that TF containing microparticles derived from activated monocytes, leucocytes [80] or endothelial cells [81, 82] fuse with the platelet

membrane through a receptor mediated process [77, 80] and thereby deliver TF into platelets. However other studies demonstrated the presence of a spliced TF mRNA in activated human platelets and confirmed an increased TF protein synthesis and activity in ADP stimulated platelets [82].

TF containing microparticles have been characterized in the plasma. Beside endothelial and vascular smooth muscle cells [69, 83-85], monocytes and platelets are the main source of TF containing microparticles [86, 87]. Despite studies demonstrating the role of microparticle derived TF in generation of thrombin *in vitro* [88], doubts remain about its contribution to thrombus formation *in vivo* [89, 90].

As mentioned previously, the full-length form of TF mRNA contains 6 exons and splicing of exon 5 results in a frame-shift mutation generating a soluble alternatively spliced TF protein lacking the transmembrane domain [19]. Apart from plasma, alternatively spliced TF has been found in several tissues and cell types including the lung, placenta, vascular smooth muscle cells, and keratinocytes. Whereas it remains unclear whether alternatively spliced TF exerts pro-coagulant activity [91] more recent reports have revealed a role of alternatively spliced TF in angiogenesis by promoting endothelial cell migration and differentiation [92, 93].

c. Tissue factor in the heart

In contrast to skeletal myocytes, cardiac myocytes express the full length form of TF under basal conditions [94, 95]. Studies performed in mice expressing low levels of TF decrypted a potential role of TF in the maintenance of heart hemostasis. Mice expressing low-TF had increased cardiac hemorrhages and hemosiderin depositions ultimately leading to cardiac fibrosis and a reduction in left ventricular function as compared to wild type animals [96]. Phagocytosis of hematin derived from hemorrhaging erythrocytes is thought to play a key role in this process. Interestingly

mice lacking factor IX had normal hearts, underlining the specific role the TF:VIIa complex in hemostatic protection of the heart.

2. Tissue factor in cardiovascular diseases

a) Implications in thrombus formation

Although TF is critically involved in initiation of the coagulation cascade, questions regarding the role and contribution of the different TF pools in arterial thrombus formation remain open.

Under normal hemostatic conditions TF is constitutively expressed in the vascular smooth muscle layer of the media and in fibroblasts present in the vessel and the adventitia surrounding the blood vessels [97, 98]. Hence, disruption of blood vessel integrity promotes the interaction between circulating factor VII and the intravascular TF pool leading to initiation of the clotting cascade. Moreover the release of inflammatory triggers following vascular injury or plaque rupture induces the expression of TF in endothelial cells and circulating cells in the blood thereby potentiating a full coagulation response [4]. In contrast to the initiation phase of coagulation, the mechanisms underlying the propagation of the clot growth are not as evident.

The first hypothesis introduced blood-borne TF as a major contributor to the propagation of the clotting reaction. This consideration is based on the fact that the maintenance of coagulation requires a constant activity of TF as a cofactor and that the formed fibrin clot isolates and prevents the initially formed TF:VIIa complexes from reacting with circulating new inactive coagulation factors [99]. This hypothesis is supported by the observation that in the blood TF is detected at very low levels unable to trigger a coagulant response whereas the TF concentration reaches functionally significant levels on the surface of a forming thrombus [19, 100, 101]. In

addition other studies revealed the presence of embedded TF in murine [90, 102] and human [103] thrombi. In vitro, the role of blood-borne TF in coagulation is strengthen by the study of Okorie et al. who demonstrate that in contrast to no-flow conditions the subpicomolar TF concentrations found in blood cause an increase in thrombin formation under flow [104].

On the other hand, recent experiments performed in mathematical as well as whole blood models revealed different stages of TF requirement in the clotting process and showed that after the onset of thrombin generation the propagation of coagulation is TF independent [105]. By abrogating TF activity with specific antibodies targeting either VIIa or TF, Orfeo et al. distinguished 3 phases in the clotting process: a first short (<10 seconds) period of absolute TF:VIIa dependence; a second period (10-240 seconds) of partial TF:VIIa dependence that decreases with the progress of the reaction, and after 2 minutes a period which is TF:VIIa independent. In addition this study also showed that the initial activation of procoagulant proteases by the TF:VIIa complex is sufficient to maintain a pool of activated procoagulant catalysts able to maintain a sustained coagulation response in a TF independent manner.

Since venous thrombosis is not associated with vessel damage, circulating TF may play an important role in this pathology. In a model of jugular vein thrombosis inhibition of TF reduced thrombus growth in the uninjured vein [106]. However, in another study hematopoietic cell-derived TF did not affect venous thrombosis in an inferior vena cava thrombosis model in the mouse [90].

In summary, the exact role of the different TF pools and their contributions to the propagation of clot formation remains controversial and further studies are definitely required.

b) Implications in inflammation and atherosclerosis

Inflammation is tightly linked to the development of atherosclerosis [107]. Elevated levels of inflammatory cytokines known to induce TF expression, such as TNF-α and interleukins, are observed in early atherosclerotic lesions after adhesion and infiltration of monocytes to the vessel wall. In the early stages of atherogenesis, TF mRNA and antigen is expressed in macrophages [52, 108] and as the lesion progresses TF is increasingly expressed in other cell types such as foam cells, endothelial cells and smooth muscle cells. Within the necrotic core [109] it is predominantly associated with TF containing microparticles synthetized by foam cells, monocytes, lymphocytes and smooth muscle cells and encountered in the extracellular space [110]. During plaque rupture the content of the core comes in contact with the circulating blood and TF originated from the plaque content and from activated endothelial and smooth muscle cells triggers thrombus formation and thereby acute vascular events. Indeed higher levels of TF are described in the atherosclerotic plaques of patients suffering acute coronary syndromes as compared with patients suffering stable angina [111].

In turn, recent studies indicate that TF:VIIa signaling may play a role in the pathogenesis of atherosclerosis. The TF:VIIa complex has been shown to be a strong chemotactic stimulus for vascular smooth muscle cells [112]. Inhibition of TF:VIIa by overexpression of tissue factor pathway inhibitor lead to a consecutive reduction of vascular smooth muscle cell migration in vitro and in vivo impaired vascular remodeling in an mouse model [113, 114]. Since TF:VIIa mediated cell signaling induces a number of gene products involved in cell proliferation and migration, it is very likely similar effects occur within TF expressing cells in atherosclerotic lesions. As mentioned in the previously TF:VIIa signaling enhances the expression of the connective tissue growth factors CCN1 and CCN2 in fibroblasts and vascular smooth muscle cells.[39] Interestingly, similar to TF, CCN1 and CCN2 mRNA levels are highly

expressed in atherosclerotic lesions of apoE-/- mice and in human atherosclerotic plaques [115, 116]. Hence, it is conceivable that by inducing the expression of CCN1 and CCN2, the TF:VIIa complex promotes plaque progression via enhanced extracellular matrix accumulation and vascular smooth muscle cell proliferation. In addition a role of CCN1 and CCN2 in monocyte and platelet adhesion to the subendothelial matrix after endothelial injury has also been described [117, 118].

In response to inflammatory mediators TF transcription can be induced in different cell types. Vice versa, recent results show that TF on these cells appears to regulate the inflammatory response. During sepsis the TF pathway induces a lethal inflammatory exacerbation independently of thrombin generation and clot formation [119, 120]. Inhibition of the TF:VIIa complex was paralleled by a decrease in interleukin secretion and a reduction in inflammatory infiltrations in several tissue during sepsis [121]. This finding is confirmed by the observation that in a model of septicemia mice expressing low TF levels display reduced interleukin levels and an increased survival compared with control mice [122]. Studies performed in PAR2 deficient mice suggest that the pro-inflammatory TF:VIIa signaling response is likely mediated via PAR2 [122]. However it is worth noting that deficiency of PAR1 or PAR2 alone has no effect and that reduction in inflammation and mortality is only observed in the context of a combined inhibition of thrombin generation and PAR2 deficiency, indicating a tight link between coagulation and inflammation.

c) Implications in acute coronary syndromes

Higher levels of TF have been detected in coronary atherectomy specimens from patients with unstable angina or myocardial infarction [123]. In line with this, plasma TF levels are elevated in patients with acute coronary syndromes compared with controls [124, 125] and have been shown to correlate with an unfavorable outcome in

those patients [126]. In addition, patients with unstable angina or non-ST-elevation myocardial infarction and a high TIMI score (≥4) show increased TF plasma levels compared with those with a low TIMI score (<3) [127].

Percutaneous transcoronary angioplasty and consecutive stent implantation has become the gold standard in the treatment of stenotic lesions in acute coronary syndromes. The local inflammatory reaction following balloon dilatation and stent implantation [128] induces local TF release from the dissected plaque. Thus, mostly due to promigratory effects of TF on vascular smooth muscle cells, elevated levels of TF have been reported to positively correlate with the incidence of restenosis after percutaneous interventions [129]. Despite the reduction in restenosis following deployment of drug eluting stents (DES) when compared to bare metal stents, stent thrombosis rates have not been decreased with the use of DES and may even be higher [130]. One possible explanation may be that the drugs eluting from the stents which are intended to inhibit restenosis by reduction of vascular smooth muscle cell migration and proliferation also exert biological effects on other cell types in the vasculature, in particular endothelial cells. For instance recent studies revealed that drugs coating the first and second generation stents, like rapamycin, everolimus or zotarolimus, enhance TF expression in endothelial cells and accelerate arterial thrombus formation via inhibition of the mTOR/p70S6K pathway [49, 131]. Similarly, paclitaxel another widespread stent coating drug was shown to increase endothelial TF expression and activity through activation of the MAP kinase JNK [132]. Hence, these findings may raise important issues in the context of drug eluting stent design since coating a stent with a drug exerting not only antiproliferative but also antithrombotic properties may offer new options in the interventional treatment of vascular disease.

d) Tissue factor and cardiovascular risk factors

Diabetes mellitus, independently of coronary disease, is associated with an increase in plasmatic TF activity [133], TF antigen levels [134] and TF containing microparticles [135]. In line with this observation improvement of glycemic control or application of oral antidiabetics such as the peroxisome-proliferator-activated receptor-γ agonist rosiglitazone downregulate TF protein expression in these patients [136]. Consistent with this observation, high glucose increases TF antigen and activity in monocytes derived from healthy subjects [137, 138]. A similar effect of high glucose on TF expression in human endothelial cells was also observed [139]. In diabetes, hyperglycemia induces the formation of advanced glycation end-products [78], which upregulate TF expression in endothelial cells and monocytes via nuclear factor kappa B [140-142]. Consistent with these findings, higher levels of AGE and TF were observed in the vasculature of diabetic mice. Blockade of the corresponding receptor for AGE however significantly reduced the TF increase in the aorta of these mice[140].

Patients with hyperlipidemia display an increased risk of developing thrombosis[143]. Elevated low density lipoprotein (LDL) levels are associated with an increased TF plasma activity [144]. Oxidized LDL triggers TF protein synthesis in monocytes [145], endothelial cells [146] and vascular smooth muscle cells [147]. In contrast, high-density lipoproteins (HDL) reduce thrombin-induced TF expression in endothelial cells through PI3K pathway activation [148]. 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors (statins) reduce TF expression in monocytes [149], endothelial cells [46] and vascular smooth muscle cells [150]. These findings are confirmed in studies performed in ApoE -/- mice in which simvastatin and rosuvastatin treatment reduced the TF antigen expression in atherosclerotic lesions significantly independent of lipid lowering effects [151, 152]. Fibrates, which are also in use as

lipid lowering drugs in the treatment of hyperlipidemia, also exert pleiotropic effects on vascular TF expression beyond their lipid lowering properties [153].

In contrast to normotensive subjects, hypertensive patients display increased plasma levels of TF antigen [154] which can be lowered by an appropriate antihypertensive management [155]. Several mechanisms are thought to mediate this enhanced TF expression observed in arterial hypertension. For instance the chronic exposure to shear stress induces the activation of transcription factors and consequently the expression of TF in endothelial cells [156]. On the other hand angiotensin II was shown to increase TF expression in monocytes [157], endothelial cells [156] and vascular smooth muscle cells via the angiotensin II type 1 receptor. Consistent with this observation ACE inhibitors and angiotensin II type 1 receptor antagonists reduce TF plasma activity in hypertensive patients and inhibit endotoxin-induced TF expression in monocytes [158].

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VI. Original articles

Dietary Alpha-Linolenic Acid Inhibits Arterial Thrombus Formation, Tissue Factor Expression, and Platelet Activation

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Abstract

Objective. Plant-derived *alpha*-linolenic acid (ALA) may constitute an attractive cardioprotective alternative to fish-derived *n*-3 fatty acids. However, the effect of dietary ALA on arterial thrombus formation remains unknown.

Methods and Results. Male C57Bl/6 mice were fed a high ALA or low ALA diet for 2 weeks. Arterial thrombus formation was delayed in mice fed a high ALA diet as compared to those on a low ALA diet (n=7; $p<0.005$). Dietary ALA impaired platelet aggregation to collagen and thrombin (n=5; $p<0.005$) and decreased p38 MAP kinase activation in platelets. Dietary ALA impaired arterial tissue factor (TF) expression, TF activity and NFκB activity (n=7; $p<0.05$); plasma clotting times and plasma thrombin generation did not differ (n=5; p=NS). In cultured human vascular smooth muscle and endothelial cells ALA inhibited TF expression and activity (n=4; $p<0.01$). Inhibition of TF expression occurred at the transcriptional level via the MAP kinase p38 in smooth muscle cells and p38, ERK1/2, and JNK1/2 in endothelial cells.

Conclusions. ALA impairs arterial thrombus formation, TF expression, and platelet activation, and thereby represents an attractive nutritional intervention with direct dual anti-thrombotic effects.

Key words: alpha-linolenic acid; thrombosis; platelets; tissue factor, MAP kinases

Arterial thrombosis is the critical step in the development of acute vascular syndromes.[1,2,3] Circulating platelets are activated by interaction with subendothelial collagen at the site of an injury.[4] In parallel, membrane bound tissue factor (TF) expressed on vascular cells acts as a receptor for activated factor VII (VIIa).[5,6] The TF/VIIa complex triggers the coagulation cascade and the formation of activated factor X (Xa) ultimately resulting in thrombin formation, which in turn cleaves protease-activated receptors on the platelet surface boosting platelet activation and clot formation[4]. The critical role of platelets and TF in the pathogenesis of acute coronary syndromes has indeed been well documented.[7,8,9]

Experimental and epidemiological studies have extensively characterized the cardioprotective and anti-thrombotic effects of fish-derived dietary long chain *n*-3 fatty acids eicosapentaenoic acid (C20:5 *n*-3, EPA) and docosahexaenoic acid (C22:6 *n*-3, DHA).[10,11,12,13] Consistent with these observations, an increased risk for developing cardiovascular disease was identified in populations with low intake of EPA and DHA, prompting the American Heart Association (AHA) to modify their nutrition guidelines.[14]

ALA is an essential *n*-3 FA found at high concentrations in vegetable oils, in particular flaxseed oil, where it accounts for 50% of the total FA content. Following oral intake, ALA is partially converted into EPA and DHA. The rate of conversion of ALA into long chain *n*-3 fatty acids ranges from 0.05 to 10%.[15] In recent epidemiological studies, ALA consumption has been inversely associated with the incidence of myocardial infarction, sudden cardiac death, and coronary artery calcification.[16-20] Despite these promising data, the precise biological mechanisms mediating the cardioprotective effects of ALA remain barely understood. In particular, it remains controversial whether the rather poor conversion of ALA to EPA and DHA can account for the beneficial effects of ALA or whether ALA exerts direct biological

effects.[21,22]

Since increasing evidence suggests that ALA can serve as a cardioprotective nutritional supplement, this study addresses the question whether dietary ALA inhibits thrombus formation *in vivo* and analyzes the mechanisms involved.

Materials and Methods

For the detailed Materials and Methods please see the supplemental Materials and Methods (available online at http://atvb.ahajournals. org).

ALA diet and carotid artery thrombosis model

C57BL/6 mice were fed a 0.21% w/w cholesterol diet containing either a high ALA (7.3%) or low ALA concentration (0.03%, supplemental table 1).[23] Photochemical injury model was performed after 2 weeks of treatment.

Immunohistochemistry

Thrombus composition was analysed in paraffin fixed sections from occluded carotid arteries. Sections were stained for TF, fibrin, and the platelet marker CD41.

Prothrombin time (PT) and activated partial thromboplastin time (aPTT)

Plasma was isolated from citrated blood (3.2%; 1/10) by centrifugation (2500g; 4°C; 15 minutes). PT and aPTT were assessed using the START 4 analyzer (Diagnostica Stago, France). [24]

Thrombin generation

Plasma thrombin generation was monitored by automated calibrated thrombography and the results analysed using the Thrombinoscope software (Thrombinoscope BV, Maastricht, The Netherlands).[25,26]

Platelet aggregation

Platelet aggregation was studied by whole blood impedance aggregometry (Chrono-Log, Havertown, PA, USA).

Fatty acid profile in aortic tissue

FA profiles of the pooled tissues were analyzed by gas chromatography as described.[27] Measurements were performed in duplicate and the mean value displayed.

Cell culture

Human aortic vascular smooth muscle cells (VSMC; Clonetics, Allschwil, Switzerland) and human aortic endothelial cells (HAEC; Clonetics) were cultured as described.[24] Protein expression was determined by Western blot analysis and RNA by real-time polymerase chain reaction (PCR).

Tissue factor activity

Factor Xa generation on the surface of VSMC, HAEC, or in tissues was analyzed using a colorimetric assay (American Diagnostica Inc, ACTICHROME).

TF promoter activity

TF promoter activity was measured as described.[28] A minimal TF promoter (-227 bp to +121 bp) was cloned upstream of the firefly luciferase reporter gene and a recombinant adenoviral vector was constructed. HAEC were transduced with the vector Ad5/hTF/Luc (100 pfu/cell) for 1 hour. An adenoviral vector without reporter gene (VQAd/Empty) was used as a negative control. After transduction, HAEC were grown in medium (10% FBS) for 24 hours and then serum-starved for 24 hours prior to TNF-α stimulation with or without ALA (30 μM) pretreatment. Firefly luciferase

activity was determined by luminometer (Berthold Technologies, Bad Wildbad, Germany) and normalized to the protein concentration in the lysates.

Nuclear extracts and NFκB activity

Nuclear extracts were obtained from HAEC or mouse aorta using a nuclear extraction kit (Active Motif, Carlsbad, USA) and NFκB activity measured using a TransAM NFκB p65 kit (Active Motif).

Statistics

Data are presented as mean±SEM. Statistics were performed using the GraphPad prism 4.0 Software (GraphPad Software Inc., La Jolla, CA, USA). Statistical analysis was performed using 2-tailed unpaired Student´s t-test or one-way ANOVA as appropriate. A value of $p<0.05$ was considered significant.

Results

Dietary ALA inhibits arterial thrombosis

C57BL/6 mice were fed for 2 weeks a 0.21% cholesterol diet containing either 7.3% w/w ALA (high ALA, treated group) or 0.03% w/w ALA (low ALA, control group). No difference in body weight was observed after 2 weeks of diet (n=7; *p*=NS; data not shown). Aorta from animals fed a high ALA diet showed markedly increased ALA levels (14.9% of total FA content; supplemental table 2) as compared to tissue from control animals (0.14% of total FA content; supplemental table 2). In contrast, EPA and DHA levels did not differ between the two groups. Total cholesterol levels were not altered (control: 2.27±0.17 mmol/l; low ALA: 1.95±0.21 mmol/l; high ALA: 2.29±0.30 mmol/l; n=8; *p*=NS).

Mice on control diet developed carotid artery thrombosis within a mean occlusion time of 40±4.2 minutes, while mice on high ALA diet occluded after 68±6 minutes (n=7; $p<0.005$; figure 1A). Initial blood flow did not differ between the two groups (n=7; *p*=NS). Intravenous administration of an inhibitory anti-TF antibody before the onset of photochemical injury reduced the difference in occlusion times between control and high ALA diet (n≥7; *p*=NS; figure 1B). Plasma clotting times (PT and aPTT) were similar in the two groups (PT: 11.1±0.2 seconds [low ALA] versus 11.5±0.4 seconds [high ALA]; n=7; *p*=NS; aPTT: 20.3±0.8 seconds [low ALA] versus 21.1±0.7 seconds [high ALA]; n=7; *p*=NS). Thrombin generation (endogenous thrombin potential [ETP] and time to peak [TTP]) did not differ between the groups (ETP: 747±94 nM/minute [low ALA] versus 818±80 nM/minute [high ALA]; n=5; *p*=NS; TTP: 6.2±0.7 minutes [low ALA] versus 4.8±0.5 minutes [high ALA]; n=5; *p*=NS). Further no difference in peak thrombin values (104.4±28.7 nM [low ALA] versus 118.8±22.9 nM [high ALA]; n=5; *p*=NS) nor in lag times (2.9±1.36 minutes

[low ALA] versus 2.1±1.3 minutes [high ALA]; n=5; *p*=NS) was observed between the groups.

To analyse differences in thrombus composition, sections from occluded carotid arteries were stained for TF, fibrin, and platelets. Although thrombi were occlusive in both groups, TF staining of in the vascular media was lower in the high ALA group as compared to the control group (figure 1C; n=4). In contrast, fibrin staining was similar in thrombi from both groups. Analysis of platelet content by immunhistochemistry for CD41 demonstrated a reduced platelet content in thrombi in the ALA treated group as compared to control (figure 1D; n=4).

Dietary ALA inhibits platelet aggregation

Thrombin induced platelet aggregation (figure 2A-D) was inhibited in mice fed a high ALA diet as compared to control platelets (maximal aggregation: n=5, $p<0.005$, figure 2B; area under the curve: n=5; $p<0.005$, figure 2C; lag time: n=5, $p<0.05$, figure 2D). When aggregation was induced by collagen, a similar inhibition was observed (maximal aggregation: n=5, $p<0.005$, figure 2F; area under the curve: n=5, $p<0.05$, figure 2G; lag time: n=5, $p=0.14$, figure 2H). Platelet number did not differ significantly between the groups ($1062\pm46x10^3$/µL [low ALA] versus $911\pm5x10^3$/µL [high ALA]; n=5; *p*=NS).

Treatment of human platelets with ALA (30 µM) for 30 minutes *ex vivo* exerted a similar inhibition of thrombin and collagen induced platelet aggregation (n=5; supplemental table 3A-B). ALA (30 µM) impaired collagen and thrombin induced p38 phosphorylation (n=3; $p<0.01$ versus collagen or thrombin alone; supplemental figure 1A-B).

Dietary ALA inhibits TF expression and NFκB activation

TF activity in carotid arteries was markedly decreased in mice fed a high ALA diet as compared to controls (n=7; $p<0.005$, figure 3A). Inhibition of TF with a specific antibody confirmed that factor Xa generation was TF dependent (figure 3B). This decrease in TF activity was paralleled by an impaired TF mRNA expression in aortic tissue (n=7; $p<0.01$; figure 3C). In contrast, expression of tissue factor pathway inhibitor (TFPI) mRNA did not differ ($\Delta\Delta$Ct: 0.03±0.04 low ALA group versus 0.03±0.04 high ALA; n=5; p=NS). NFκB p65 DNA binding affinity was measured in nuclear extracts from aortic tissue. NFκB activity was impaired in mice fed a high ALA diet as compared to controls (OD 490 nm: 0.97±0.02 [low ALA] versus 0.70±0.09 [high ALA]; n=7; $p<0.05$, figure 3D).

ALA inhibits TF protein expression in VSMC

Treatment with ALA (30 μM) for 24 hours reduced TF expression in VSMC by 46% (n=4; $p<0.05$) as compared to control (figure 4A). Real time PCR analysis confirmed that ALA inhibited TF expression in VSMC at the transcriptional level (n=4; $p<0.005$; figure 4B). In line with this observation, ALA decreased TF activity in VSMC (n=4; $p<0.001$; figure 4C). Experiments performed with an inhibitory anti-TF antibody confirmed that factor Xa generation in VSMC was TF dependent (figure 4D). TF expression was neither affected by the *n*-6 FA alpha-linoleic acid (LA) nor the saturated FA stearic acid (SA) (n=4; *p*=NS; data not shown). No cytotoxic effects of ALA, LA, or SA in VSMC were observed for any of the concentrations (data not shown).

ALA inhibits p38 phosphorylation and NFkB activity in VSMC

Analysis of the MAP kinase phosphorylation demonstrated that treatment of VSMC with ALA for 24 hours resulted in a significant decrease in p38 activation

(49±14% inhibition; n=4; p<0.05; figure 5A). In contrast, activation of JNK and ERK remained unaffected. Blockade of the MAP kinase p38 with SB203580 (10 μM) inhibited TF expression in VSMC (n=4; p<0.01; figure 5B). Treatment with ALA (30 μM) reduced NFκB p65 DNA binding affinity in nuclear extracts from VSMC (n=4; *p*<0.05; figure 5C).

ALA inhibits TNF-α induced TF mRNA expression via MAP kinase and ASK1 in HAEC

HAEC were treated with ALA or vehicle for 1 hour and then stimulated with TNF-α (5 ng/mL) for 4 hours. ALA inhibited TNF-α induced endothelial TF expression in a concentration dependent manner with a maximal effect at 30 μM (86% inhibition; n=5; *p*<0.001 versus TNF-α alone; figure 6A). In line with this observation, ALA decreased TF activity in HAEC (51% inhibition versus TNF-α alone; n=4; *p*<0.001; figure 6B).

Real-time PCR analysis demonstrated that ALA (30 μM) inhibited TNF-α induced endothelial TF mRNA expression (n=4; *p*<0.001; Figure 6C). This decrease in TF mRNA was paralleled by an impaired activation of the MAP kinases JNK, p38, and ERK (28%, 62%, and 55% inhibition versus TNF-α alone, respectively; n=4; *p*<0.05; figure 6D). Consistent with this pattern of MAP kinase inhibition, ALA impaired activity of the MAPKKK ASK1 (n=3; *p*<0.005 versus TNF-α alone; figure 6E). ALA (30 μM) inhibited TNF-α induced TF promoter activation in HAEC by 47% (n=4; *p*<0.05, figure 6F). ALA (30 μM) blunted TNF-α induced IκB-α degradation (n=3; *p*<0.05). This effect was paralleled by reduced DNA binding activity of the NFκB subunit p65 (54% inhibition versus TNF-α alone, n=4; *p*<0.01). To confirm the involvement of NFκB in TNF-α induced endothelial TF expression, the effect of different NFκB inhibitors was assessed. Both BAY 11-7082 (5 μM) and PDTC (10

μM) inhibited TNF-α induced TF expression (-66% and -47% versus TNF-α alone, respectively; n=4; $p<0.01$).

Discussion

Variations of the dietary *n*-3/*n*-6 ratio have been reported to affect the susceptibility to thrombosis in atherosclerotic mice.[29] However, it remains unknown whether ALA can exert such an effect and whether if influence thrombosis directly or via other PUFA.

The present study demonstrates that dietary supplementation with ALA for 2 weeks impaired arterial thrombus formation, platelet activation, TF activity, and NFκB activity in mice in vivo. The ALA rich diet increased tissue levels of ALA, while there was no significant change in tissue levels of the long chain *n*-3 FA EPA and DHA. Consistent with these findings, ALA by itself impaired platelet aggregation and TF induction in different human primary cells cultures. This effect occurred at the transcriptional level involving inhibition of MAP kinase phosphorylation, NFκB activation, and promoter activation.

The ALA concentrations used for experiments in this study are in a clinically relevant range. Indeed, baseline plasma levels of ALA have been reported to range from 17-19 μM, whereas a daily oral ingestion of 3 g ALA leads to ALA plasma levels of 32±17 μM and is well tolerated[15]. Dietary supplementation of ALA for 2 weeks was sufficient to markedly increase the ALA levels in the aortic wall, whereas EPA and DHA levels did not differ from the control group. The lack of a measurable conversion to long chain *n*-3 FA is in line with previous epidemiological studies demonstrating that dietary ALA poorly correlates with EPA and DHA levels in adipose tissue, erythrocytes or plasma[16]. Even more important, the *in vivo* effects of dietary ALA

observed in the present study were mimicked by exogenous ALA in both human platelets and vascular cells *ex vivo*, where hepatic conversion of ALA to EPA and DHA does not occur. Taken together, these experiments indicate that the anti-thrombotic effects of ALA *in vivo* did not depend on the conversion of ALA to long chain *n*-3 FA and provide strong evidence for a direct biological effect of ALA.

To exclude possible effects of other FA, control experiments were performed with the *n*-6 FA linoleic acid as well as the saturated FA stearic acid. Neither of these FA altered TF expression, supporting the interpretation that the biological effects of the high ALA diet on TF and thrombosis occur due to the increased ALA levels.

Dietary ALA supplementation significantly delayed arterial thrombus formation triggered by photochemical injury *in vivo*. Since this effect was paralleled by a reduced platelet activation and a diminished arterial TF activity, ALA impairs thrombus formation by a dual action on both critical events involved in arterial thrombosis following vascular injury. In order to determine the relative contribution of platelets and TF to thrombus formation, an inhibitory anti-TF antibody was applied. Treatment with this antibody reduced the difference in occlusion times between the two groups, suggesting that the inhibitory effect of ALA on TF exerts a major effect on thrombus formation, while its effect on pletelets contributes to inhibition of thrombosis. These results support previous observations demonstrating that arterial thrombosis is primarly driven by TF derived from the vessel wall in this model.[30,31] TF is barely expressed under basal conditions in endothelial cells, while it is constitutively expressed in VSMC.[32] Immunohistochemical analysis confirmed that TF expression in the arterial tunica media was decreased in carotid arteries derived from the ALA treated group. Inhibition of TF was confirmed *in vivo*, since the ALA rich diet decreased TF expression and NFkB activity in arterial lysates. Consistent with these in vivo observation, treatment of VSMC with ALA for 24 hours decreased basal TF

expression. This effect was mediated at the transcriptional level via the MAP kinase p38. Pharmacological inhibition of p38 confirmed the crucial role of p38 activation in constitutive TF expression in VSMC. In line with p38 inhibition as well as with previous data obtained in monocytes and macrophages ALA also reduced NFkB activity in VSMC.[33,34] Hence, direct inhibition of TF in VSMC via p38 and NFkB seems to play a major role in ALA's effect on arterial thrombus formation.

In order to study whether ALA also affects inducible TF expression, additional experiments were performed in cytokine activated endothelial cells. TNF-α induced endothelial TF expression is mediated through activation of MAP kinases leading to activation of transcription factors such as AP-1 and EGR-1, whereas the IκB kinase pathway promotes NFκB activation.[35,36] ASK1 is a redox regulated kinase playing an essential role in stress induced activation of MAP kinases, thus triggering various cellular processes including inflammatory responses and TF expression.[32,37] In endothelial cells, TNF-α has been shown to activate ASK1 as well as MAP kinases.[6,37] Treatment with ALA significantly reduced phosphorylation of the MAP kinases p38, JNK, and ERK in endothelial cells. Consistent with the inhibition of MAP kinase phosphorylation, ALA also prevented activation of ASK1. Thus, it is likely that ASK1 represents a common upstream target of ALA disrupting the MAP kinase signalling pathway in endothelial cells.

Immunohistochemical analysis of CD41 (platelet glycoprotein IIb) expression demonstrated that thrombi contained less platelets in the ALA treated group as compared to the control group. Platelet aggregation was analyzed using collagen and thrombin as agonists, since they play a dominant role in platelet activation following vascular injury.[3] ALA impaired both collagen and thrombin induced platelet aggregation, indicating that ALA inhibits glycoprotein VI and Ib-V-IX as well as protease-activated receptor mediated platelet activation; hence, ALA does not

interfere with activation of a single pathway. Since the MAP kinase p38 plays a crucial role in collagen as well as thrombin induced platelet activation and adhesion, the effect of ALA on p38 phosphorylation in isolated platelets was investigated.[38,39] ALA decreased collagen and thrombin induced p38 activation, an effect that may well account for the reduced aggregation observed in ALA treated platelets. Taken together, these observations may provide an explanation for the remaining difference in occlusion times observed between the two groups after treatment with an inhibitory anti-TF antibody.

In summary, this study provides solid evidence for a potent dual anti-thrombotic effect of an ALA rich diet by reducing platelet activation and impairing vascular TF expression. These effects were reproduced in a mouse model as well as in human primary cell cultures and were not related to an increase in EPA and DHA levels as confirmed by our data. Hence, this study generates pathophysiological evidence for direct anti-thrombotic effects of dietary ALA supplementation. Since the limited availability or unfavourable geographic conditions restrict the access to *n-3* FA from marine origin in many countries, plant-derived ALA might therefore represent an attractive cardioprotective alternative. However, keeping in mind previous clinical setbacks with experimentally promising nutritional compounds such as vitamin E, there is a great need for placebo-controlled randomized large-scale clinical trials to confirm the long-term anti-thrombotic potential of dietary supplemented ALA.

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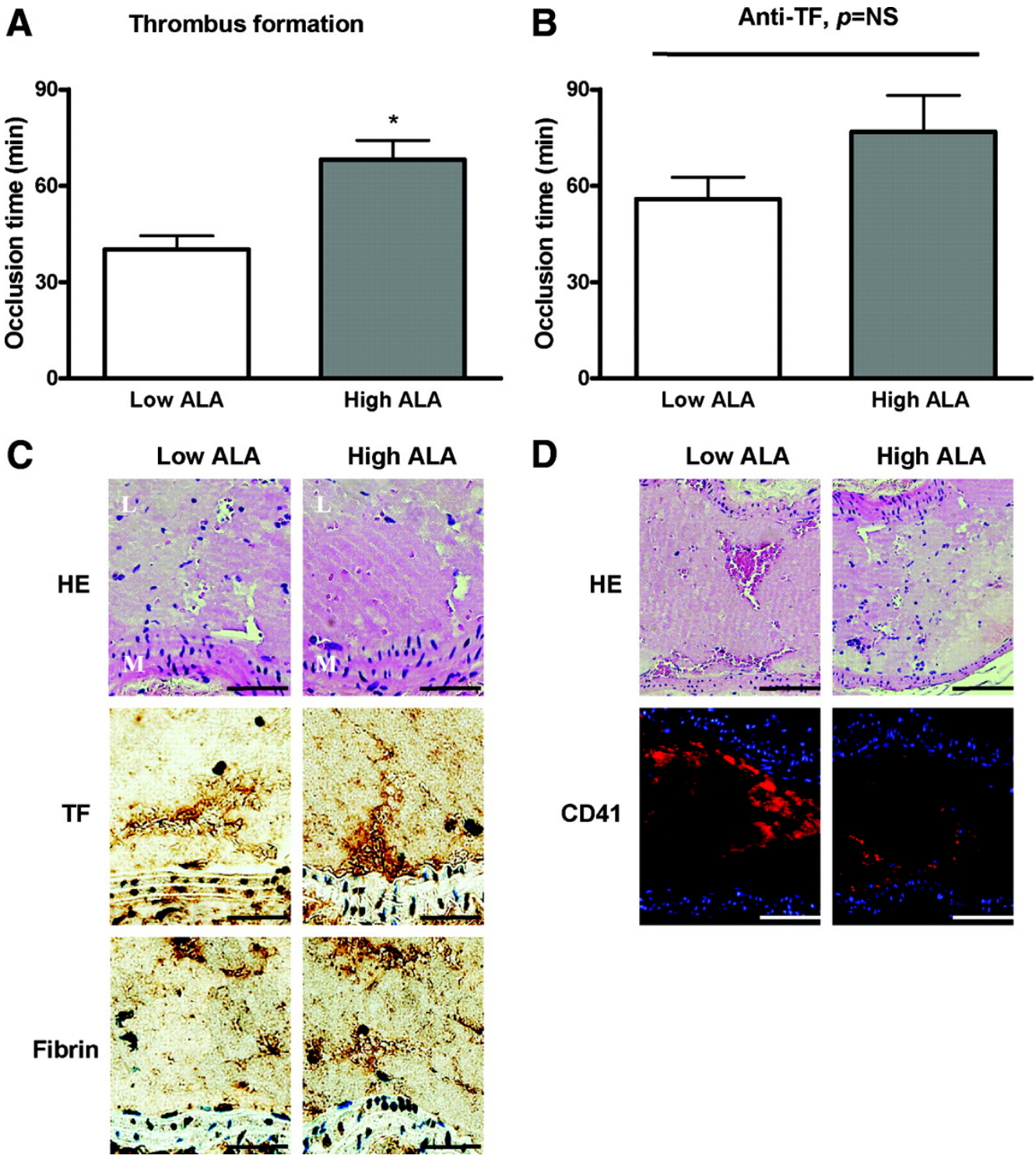

A
Thrombus formation
Occlusion time (min)
90
60
30
0
*
Low ALA
High ALA
B
Anti-TF, p=NS
Occlusion time (min)
90
60
30
0
Low ALA
High ALA
C
Low ALA
High ALA
HE
TF
Fibrin
L
M
D
Low ALA
High ALA
HE
CD41

Figure 1

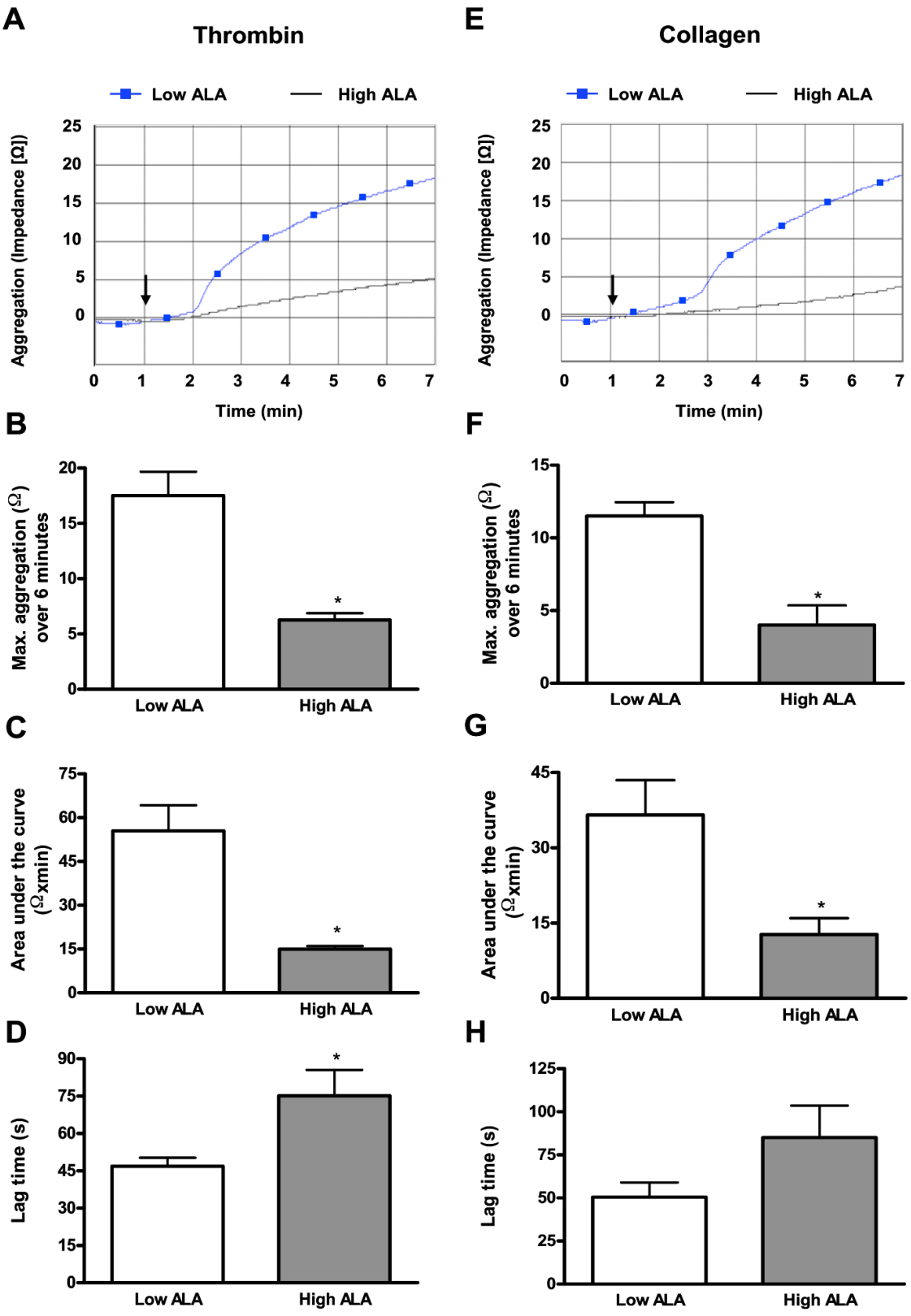

A
Thrombin
Low ALA
High ALA
Aggregation (Impedance [Ω])
Time (min)
E
Collagen
Low ALA
High ALA
Aggregation (Impedance [Ω])
Time (min)
B
Max. aggregation (Ω) over 6 minutes
Low ALA
High ALA
F
Max. aggregation (Ω) over 6 minutes
Low ALA
High ALA
C
Area under the curve (Ωxmin)
Low ALA
High ALA
G
Area under the curve (Ωxmin)
Low ALA
High ALA
D
Lag time (s)
Low ALA
High ALA
H
Lag time (s)
Low ALA
High ALA

Figure 2

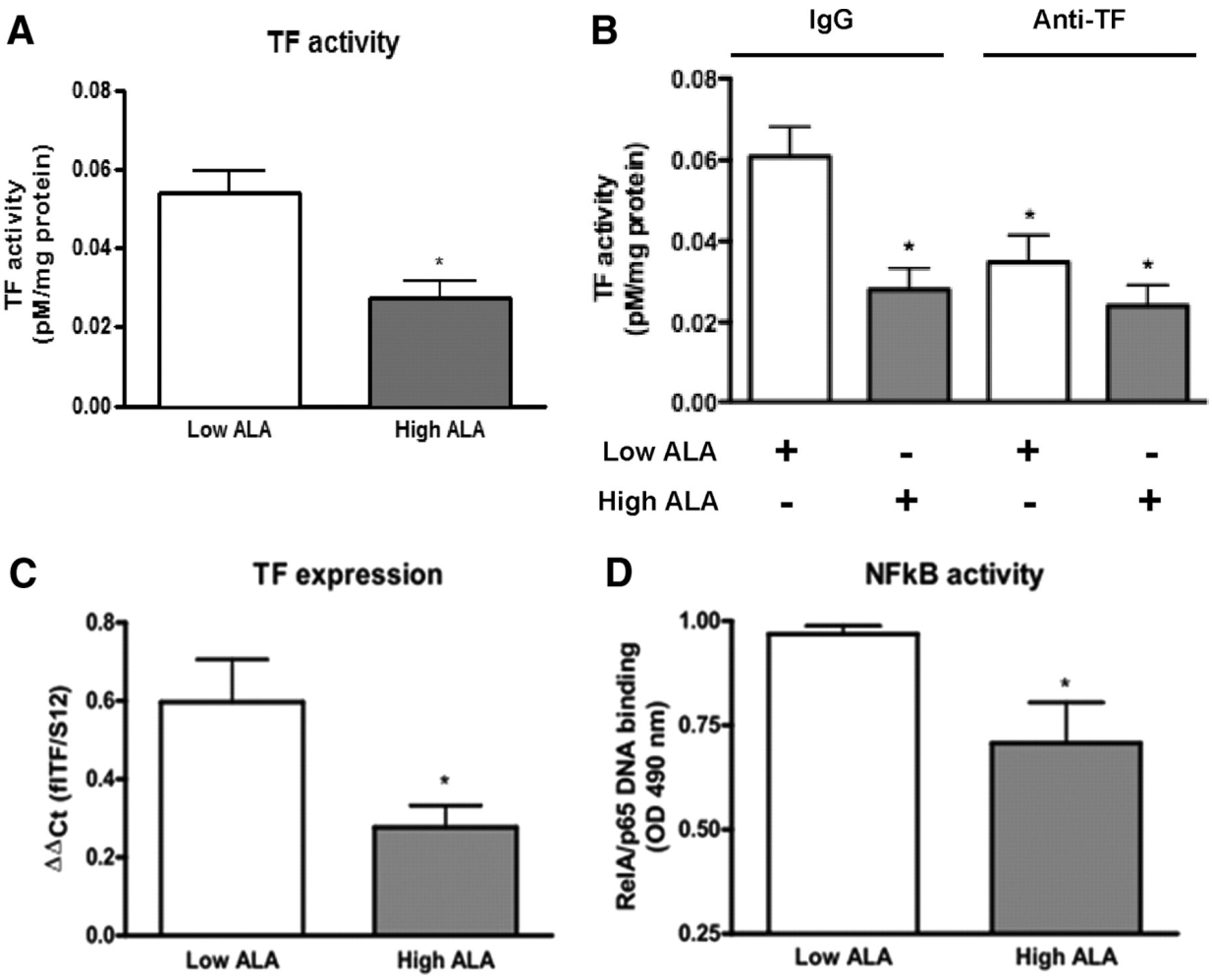

A
TF activity
TF activity (pM/mg protein)
0.08
0.06
0.04
0.02
0.00
*
Low ALA
High ALA
B
IgG
Anti-TF
TF activity (pM/mg protein)
0.08
0.06
0.04
0.02
0.00
*
*
*
Low ALA + - + -
High ALA - + - +
C
TF expression
ΔΔCt (flTF/S12)
0.8
0.6
0.4
0.2
0.0
*
Low ALA
High ALA
D
NFkB activity
RelA/p65 DNA binding (OD 490 nm)
1.00
0.75
0.50
0.25
*
Low ALA
High ALA

Figure 3

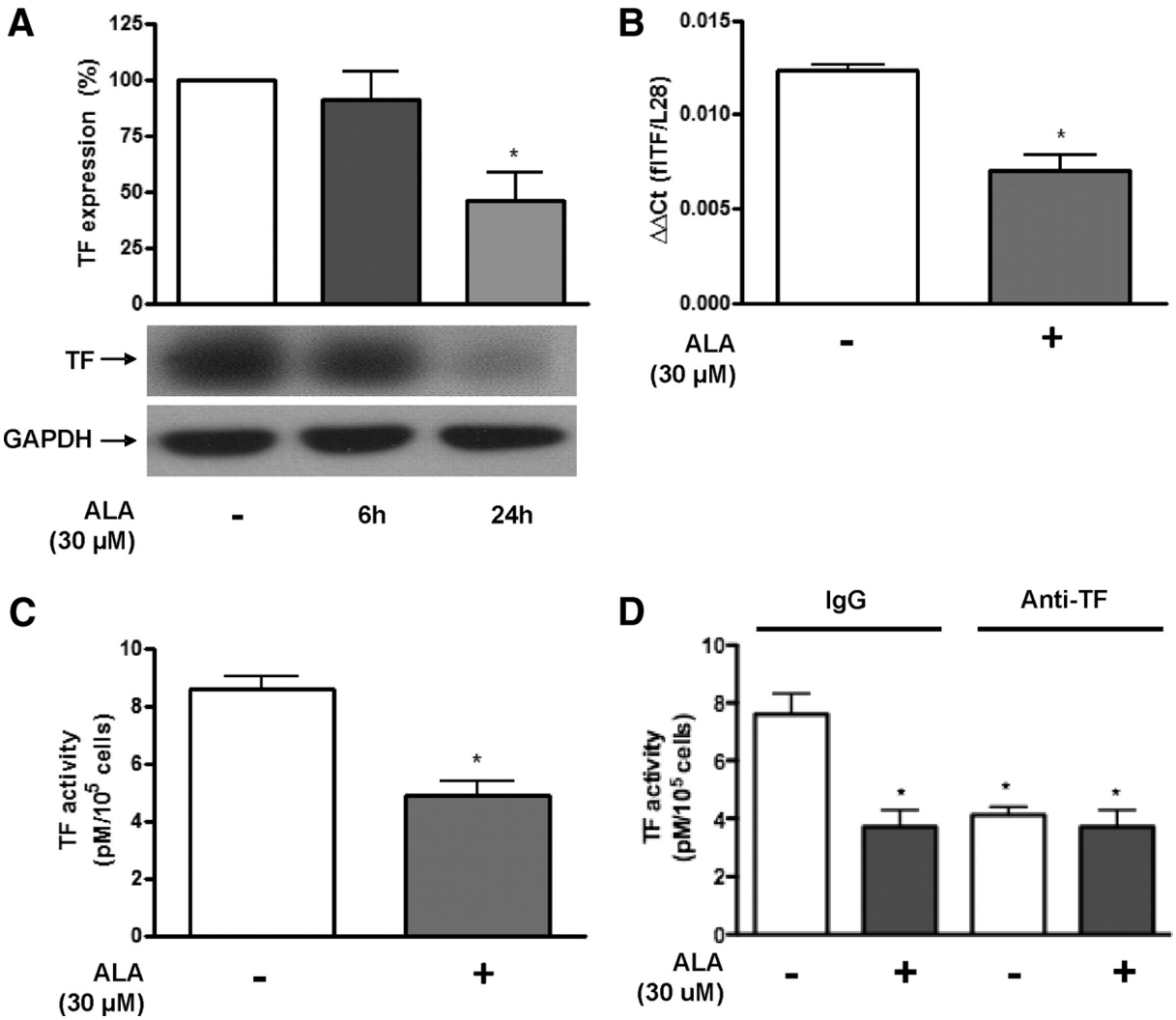

A
125
100
75
50
25
0
TF expression (%)
*
TF
GAPDH
ALA
(30 μM)
-
6h
24h
B
0.015
0.010
0.005
0.000
ΔΔCt (fITF/L28)
*
ALA
(30 μM)
-
+
C
10
8
6
4
2
0
TF activity
(pM/10^5 cells)
*
ALA
(30 μM)
-
+
D
IgG
Anti-TF
10
8
6
4
2
0
TF activity
(pM/10^5 cells)
*
*
*
ALA
(30 uM)
-
+
-
+

Figure 4

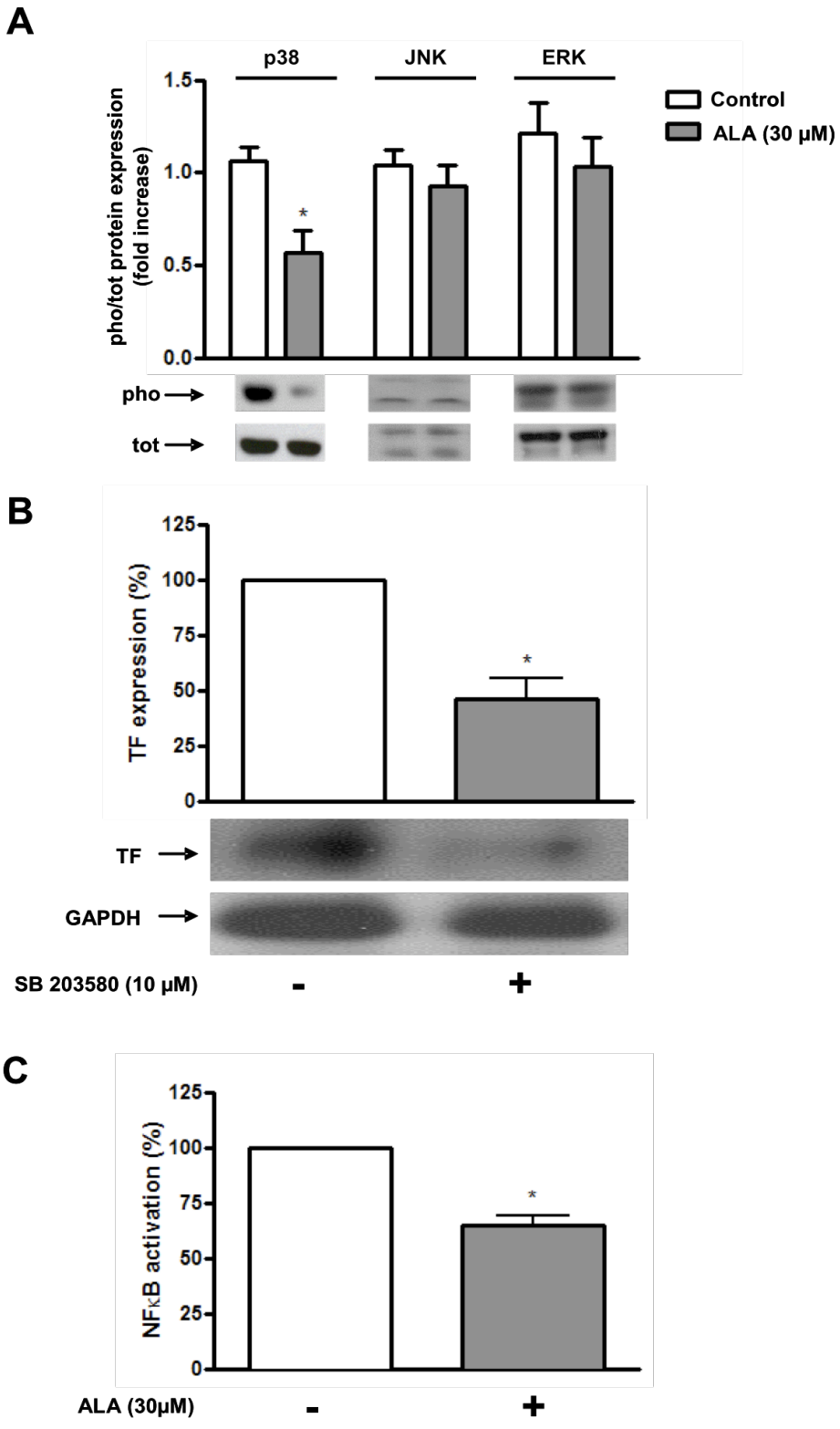

A
p38
JNK
ERK
Control
ALA (30 μM)
pho/tot protein expression (fold increase)
1.5
1.0
0.5
0.0
*
pho
tot
B
TF expression (%)
125
100
75
50
25
0
*
TF
GAPDH
SB 203580 (10 μM)
-
+
C
NFκB activation (%)
125
100
75
50
25
0
*
ALA (30μM)
-
+

Figure 5

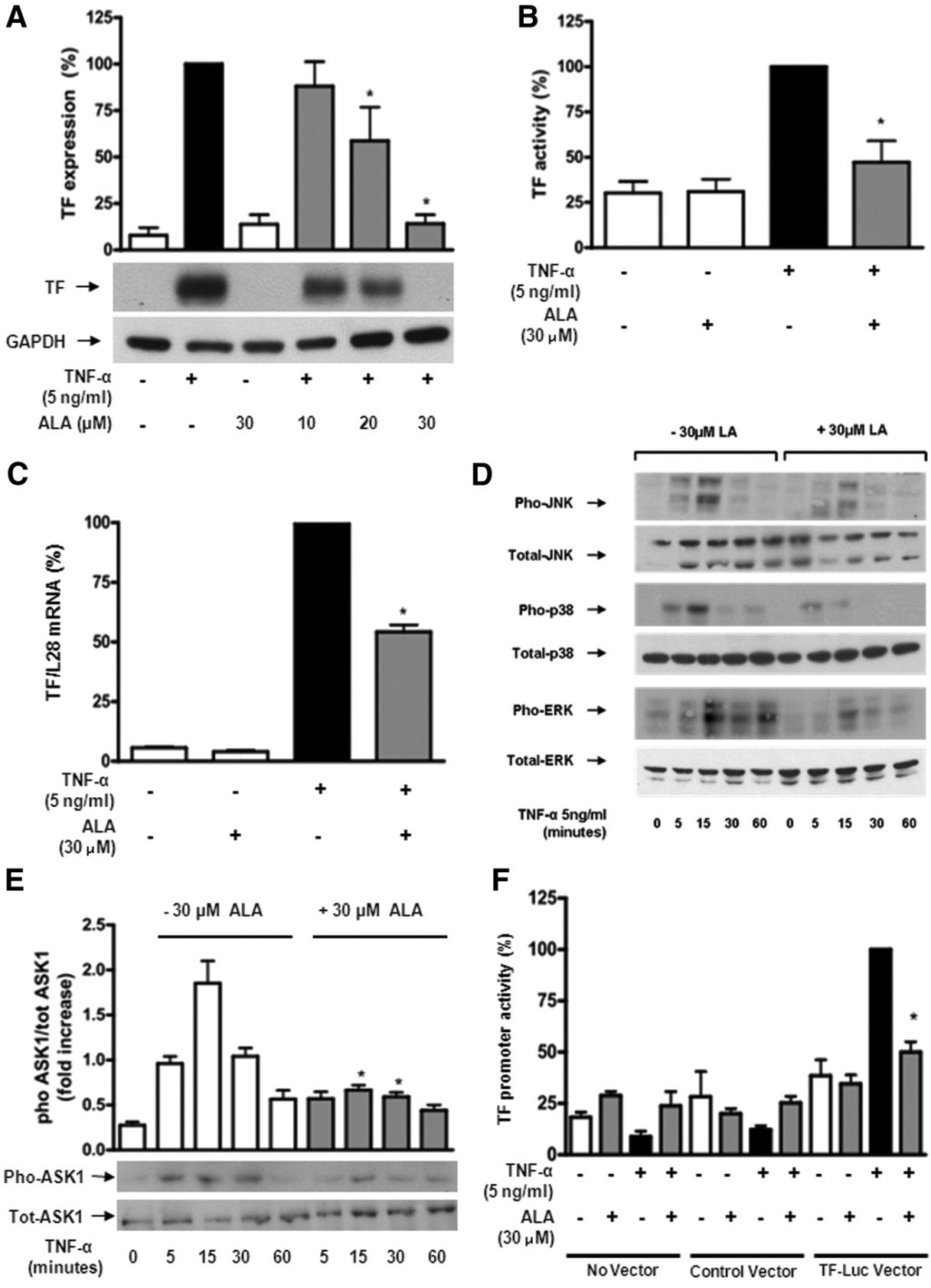

A
TF expression (%)
125
100
75
50
25
0
*
*
TF
GAPDH
TNF-α (5 ng/ml)
ALA (μM)
- + - + + +
- - 30 10 20 30
B
TF activity (%)
125
100
75
50
25
0
*
TNF-α (5 ng/ml)
ALA (30 μM)
- - + +
- + - +
C
TF/L28 mRNA (%)
100
75
50
25
0
*
TNF-α (5 ng/ml)
ALA (30 μM)
- - + +
- + - +
D
- 30μM LA
+ 30μM LA
Pho-JNK
Total-JNK
Pho-p38
Total-p38
Pho-ERK
Total-ERK
TNF-α 5ng/ml (minutes)
0 5 15 30 60 0 5 15 30 60
E
- 30 μM ALA
+ 30 μM ALA
pho ASK1/tot ASK1 (fold increase)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Pho-ASK1
Tot-ASK1
TNF-α (minutes)
0 5 15 30 60 5 15 30 60
F
TF promoter activity (%)
125
100
75
50
25
0
*
TNF-α (5 ng/ml)
ALA (30 μM)
- - + + - - + + - - + +
- + - + - + - + - + - +
No Vector
Control Vector
TF-Luc Vector

Figure 6

Figure Legends

Figure 1. Dietary ALA inhibits arterial thrombus formation.

A. Dietary ALA impairs time to thrombotic occlusion after photochemical injury in the mouse carotid artery *in vivo* (**p*<0.005). **B**. Treatment with an inhibitory anti-TF antibody blunts the difference in occlusion times (*p*=NS). **C**. Sections of thrombosed carotid artery (L: vessel lumen, M: vessel media) from control and ALA treated animals (n=4) were stained for TF and fibrin (brown stain). Scale bars equal 50 μm; HE: hematoxylin and eosin stain. **D**. Sections from occluded carotid arteries were also stained for CD41 (red stain) and nuclei (blue stain). Scale bars equal 100 μm. HE: hematoxylin and eosin stain.

Figure 2. Dietary ALA inhibits platelet aggregation.

Thrombin (**A**) and collagen (**E**) induced platelet activation is inhibited in mice fed a high ALA diet as compared to control animals. Dietary ALA reduced maximal aggregation (**B**. thrombin: **p*<0.005; **F**. collagen: **p*<0.005), reduced area under the curve (**C**. thrombin: **p*<0.005; **G**. collagen: **p*<0.05), and increased lag time (**D**. thrombin: **p*<0.05; **H**. collagen: *p*=NS).

Figure 3. Dietary ALA inhibits TF expression and NFκB activity in vivo.

Dietary ALA inhibits TF activity as assessed by FXa generation in carotid arteries (**A**. **p*<0.005). **B**. Treatment of tissue lysates with an inhibitory anti-TF antibody (10 mg/ml) inhibits FXa generation (**p*<0.01 versus control diet group in the presence of an IgG control antibody). Dietary ALA impairs TF mRNA expression (**C**. **p*<0.05),

and NFkB p65 DNA binding affinity (**D**. *p<0.05) in mouse carotid arteries.

Figure 4. ALA inhibits TF expression in VSMC.

A. Treatment with ALA (30 μM) for 24h inhibits TF protein expression in VSMC (*p<0.05 versus control). **B**. ALA (30 μM) inhibits TF mRNA expression (*p<0.005 versus control) after 24h. **C.** ALA (30 μM) also impairs TF activity in VSMC (*p<0.001). **D**. Treatment of VSMC with an inhibitory anti-TF antibody inhibits FXa generation (*p<0.05 versus control treated with an IgG control antibody).

Figure 5. ALA inhibits p38 activation and NFkB activity in VSMC.

A. Treatment with ALA (30 μM) for 24h decreases p38 activation in VSMC (*p<0.05 versus control). **B**. Inhibition of p38 with SB203580 (10 μM) for 24h impairs TF expression in VSMC (*p<0.01 versus control). **C**. ALA (30 μM) reduces DNA binding activity of the NFκB subunit p65 in VSMC (*p<0.05 versus control).

Figure 6. ALA inhibits TNF-α induced TF expression in HAEC

A. Treatment with ALA (10-30 μM) for 1h inhibits TNF-α induced TF protein expression in human endothelial cells (*p<0.01 versus TNF-α alone). **B**. ALA (30 μM) inhibits TNF-α induced endothelial TF surface activity (*p<0.001 versus TNF-α alone). **C.** ALA (30 μM) inhibits TNF-α induced endothelial TF mRNA expression (*p<0.001 versus TNF-α alone). **D.** Incubation with ALA (30 μM) for 1h inhibits TNF-α induced transient phosphorylation of JNK, p38, and ERK. **E**. ALA (30 μM) inhibits TNF-α induced ASK1 phosphorylation (*p<0.005 versus TNF-α alone). **F.** ALA (30 μM) impairs endothelial TF promoter activation (*p<0.05 versus TNF-α alone).

Table I

Diet composition

	Low ALA		high ALA	
Composition (per kg)				
	g	**kcal**	**g**	**kcal**
DL-Methionine	3	12	3	12
Casein	195	780	195	780
Corn Starch	50	200	50	200
Maltodextrin 10	100	400	100	400
Sucrose	341	1364	341	1364
Cellulose, BW200	50	0	50	0
Corn Oil	10	90	10	90
Cocoa Butter	**137**	**1233**	**0**	**0**
Flaxseed Oil	**0**	**0**	**137**	**1233**
Primex Shortening	63	567	63	567
Mineral Mix S10001	35	0	35	0
Calcium Carbonate	4	0	4	0
Vitamin Mix V10001	10	40	10	40
	% (w/w)	**kcal%**	**% (w/w)**	**kcal%**
Protein	20	17	20	17
Carbohydrate	50	43	50	43
Fat	21	40	21	40
Total kcal/kg	**4676**		**4676**	
ALA (C18:3 *n*-3; % [w/w])	**0.03**		**7.3**	

Table II

Fatty acid profile in aortas from mice fed a low and high ALA diet

	low ALA (% of FA)	**high ALA** (% of FA)
SFA	**29.42**	**25.07**
MUFA	**59.21**	**40.47**
PUFA	**11.37**	**34.46**
Total n-3	**1.69**	**16.09**
n-6/n-3	**6.41**	**1.18**
ALA	**0.14**	**14.99**
EPA	**<0.1**	**<0.1**
DHA	**0.42**	**0.46**

SFA=saturated fatty acid; MUFA=monounsaturated fatty acids, PUFA=polyunsaturated fatty acids; ALA=*alpha*-linolenic acid; EPA=eicasopentanoic acid; DHA=docosohexanoic acid

Table III

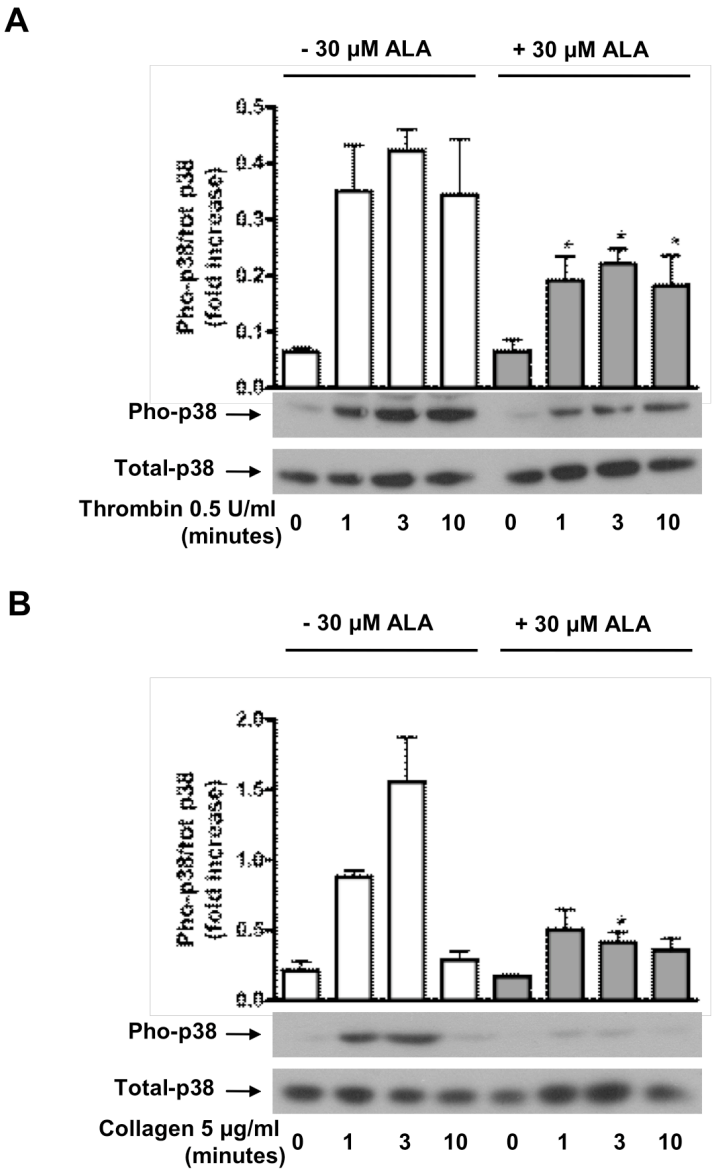
A. ALA inhibits thrombin induced human platelet activation ex vivo (n=5)

	Vehicle	30 µM ALA	*p*-value
Max. Aggregation (Ω)	28.25±1.7	17±1.8	<0.05
Area under the curve (Ωxmin)	104.4±9.8	53.3±9.3	<0.01
Lag time (seconds)	33.0±11.9	62.0±30.2	0.15

B. ALA inhibits collagen induced human platelet activation ex vivo (n=5)

	Vehicle	30 µM ALA	*p*-value
Max. Aggregation (Ω)	35.24±1.3	26±1.4	<0.005
Area under the curve (Ωxmin)	114.4±9.8	83.3±9.3	<0.01
Lag time (seconds)	18.0±4.9	34.0±4.2	<0.05

Figure I



Supplemental figure I. ALA inhibits thrombin and collagen induced p38 activation in platelets.

A. ALA (30 µM) abrogates thrombin induced p38 phosphorylation (**p*<0.01 versus thrombin alone). **B**. ALA inhibits collagen induced p38 phosphorylation (**p*<0.01 versus collagen alone).

Supplement Material

Material and methods

ALA diet and carotid artery thrombosis model

8-week-old male C57BL/6 mice weighing on average 24 g were fed a 0.21% w/w cholesterol diet containing either a high ALA (7.3% w/w , D06080702, Research Diets, New Brunswick, NJ, USA) or low ALA concentration (0.03% w/w, D06080701, Research Diets) for 2 weeks (supplemental table 1). ALA was supplemented as flaxseed oil in the high ALA group and replaced by cocoa butter in the control group. Thrombus formation was induced by photochemical injury on the 14^{th} day of diet. Mice were anesthetized by intraperitoneal injection of sodium pentobarbital (87 mg/kg; Butler, Columbus, OH). Rose bengal (Fisher Scientific, Fair Lawn, NJ, USA) was diluted in phosphate-buffered saline (PBS) and then injected into the tail vein at a final concentration of 50 mg/kg. The right common carotid artery was exposed following a midline cervical incision and the blood flow monitored using a Doppler flow probe (Model 0.5 VB, Transonic Systems, Ithaca, NY) connected to a flowmeter (Model T106, Transonic Systems). Photochemical injury was induced by a 1.5 mW green light laser (540 nm; Melles Griot, Carlsbad, CA) 6 minutes after intravenous rose bengal injection. From the onset of injury, blood flow was monitored until occlusion occurred, which was defined as a flow below 0.1 ml/min for at least 1 minute. Where mentioned, an inhibitory mouse anti-TF antibody obtained by immunization of rabbits against the extracellular murine TF amino acid domain 29-250 (American Diagnostica Inc., Stamford, CA, No. 4515,) was injected intravenously at a concentration of 3 mg/kg body weight 20 minutes prior to the rose bengal injection.

Immunohistochemistry

Occluded carotid arteries were harvested, fixed in 3% formalin and embedded in paraffin. Sections were stained for platelets (anti–mouse CD41; Abcam, Cambridge, UK; 1:50 dilution), fibrin (Anti-human, fibrin Fragment E, Cedarlane, Burlington, ON, Canada; 1:100 dilution) and TF (American Diagnostica; 1:50 dilution). Slides were blocked with 10% goat serum, and the primary antibody applied overnight at 4°C. Immunodetection was performed using biotinylated rabbit anti–mouse (for fibrin and TF, 1:200 dilution), or Texas red conjugated goat anti-rat secondary antibodies (1:200 dilution). Slides were counterstained with hematoxylin and eosin (TF and fibrin) or 4',6-diamidino-2-phenylindole (CD41) .

Platelet preparation

For analysis of p38 expression, citrated whole blood was centrifuged at 170xg for 7 minutes to obtain platelet-rich plasma (PRP). PRP was centrifuged a second time at 170xg for 7 minutes to remove residual erythrocytes. To isolate platelets PRP was centrifuged at 350xg. Pelleted platelets were resuspended in tyrode buffer (10 mM Hepes, 12 mM $NaHCO_3$,137 mM NaCl, 2.7 mM KCl, 5 mM glucose; pH 7.4) and incubated with thrombin or collagen in the presence of ALA (30 μM) or the vehicle for various time points. Reactions were stopped by addition of ice-cold lysis buffer and p38 phosphorylation assessed by western blot analysis as described below.

Platelet count and aggregation

Platelets were counted by flow cytometry using whole blood collected in EDTA tubes (B&D Diagnostics, Franklin Lakes, NJ, USA). Platelet aggregation was studied using a Chrono-Log whole blood impedance aggregometer (Chrono-Log, Havertown, PA, USA). For studies performed in murine platelets, citrated blood was drawn by

puncture from the right ventricle. For studies in human platelets, blood was obtained from healthy human volunteers and treated with ALA (30 μM; Cayman Chemical, Ann Arbor, MI, USA) or vehicle (ethanol 0.1%, Sigma Aldrich, St. Louis, USA) for 30 minutes. Aggregation studies were performed with citrated blood within 1 hour. Platelets were equilibrated under constant stirring for 1 minute prior addition of human thrombin (0.5 U/mL; Sigma Aldrich) or equine collagen type 1 (5 µg/mL; Chrono-Log). Aggregation was displayed as a function of time (AGGRO/LINK® Software; Chrono-Log). Results were monitored for 6 minutes and expressed as maximal aggregation (ohm[Ω]), area under the curve (Ωxmin), and lag time (seconds).

Fatty acid profile in aortic tissue

Aortic tissue was pooled (n=7 per group) and FA profiles of the tissues were analyzed by gas chromatography. Fat was extracted via hexane:isopropanol (3:2) and triglycerides were saponified using methanolic sodium hydroxide. FA were converted to fatty acid methyl ester (FAME) with methanolic boron trifluoride. FAME were separated using a gas chromatograph (Hewlett Packard HP 6890 Series, GC Systems, Waldbronn, Germany) equipped with a 200 mm x 0,25 mm CP7421-column (Varian, Middleburg, NL). For FAME identification, a FAME mixture was used as external standard (Supelco 37 component FAME mix). The proportion of different FAME was calculated using the ratio of the peak area of the respective FAME to the sum of total FAME peak areas. FA were evaluated using the HP ChemStation software (Hewlett Packard, CA, USA). Measurements were performed in duplicate and the mean value displayed.

Prothrombin time (PT) and activated partial thromboplastin time (aPTT)

Plasma was isolated from citrated blood (3.2%; 1/10) by centrifugation (2500xg; 4°C; 15 minutes). PT and aPTT were assessed using the START 4 analyzer (Diagnostica Stago, France).

Thrombin generation

Plasma thrombin generation was monitored by automated calibrated thrombography.[1] Platelet poor plasma was mixed with human recombinant TF (5 pM) and a fluorogenic substrate for thrombin (Z-Gly-Gly-Arg-AMC Bachem Basle, Switzerland) and then recalcified. Thrombin generation was measured in a Fluoroskan® Ascent reader (Thermo Labsystems, Helsinki, Finland) and calculated using the Thrombinoscope software (Thrombinoscope BV, Maastricht, The Netherlands). Endogenous thrombin potential, representing the area under the thrombin generation curve and time to peak thrombin formation (TTP) were displayed using the Prism 4 software package (GraphPad Software Inc., La Jolla, USA).

Cell culture

Human aortic vascular smooth muscle cells (VSMC; Clonetics, Allschwil, Switzerland) and human aortic endothelial cells (HAEC; Clonetics) were cultured as described.[2] For experiments, VSMC were serum-starved for 24 hours before pretreatment with ALA (Cayman Chemical, Ann Arbor, MI, USA), stearic acid (SA, Sigma Aldrich), linoleic acid (LA, Sigma Aldrich) or vehicle (ethanol 0.1%, Sigma Aldrich) for 6 or 24 hours. To block the mitogen-activated protein (MAP) kinase p38 MAP kinase (p38), VSMC were treated with SB203580 (Sigma Aldrich; 10) for 24 hours. For experiments with HAEC, cells were incubated with ALA for 1 hour prior stimulation with 5 ng/mL TNF-α (R&D Systems, Minneapolis, MN) for 4 hours for protein expression analysis and for 2h for analysis of TF mRNA expression. NFκB

activation was inhibited by ammonium pyrrolidinedithiocarbamate (PDTC) or BAY 11-7082 (both from Sigma Aldrich) pretreatment for 24 hours. To determine cytotoxicity, a colorimetric assay for detection of lactate dehydrogenase release was applied (Roche, Basel, Switzerland).

Western blot

Protein expressionin was determined by Western blot analysis. Antibodies against human TF (No. 4503) and tissue factor pathway inhibitor (TFPI, No. 4901) (both from American Diagnostica, Stamford, CT) were used at 1:2500 dilution. Antibodies against phosphorylated p38, ERK, JNK, and ASK1 (all from Cell Signaling) were used at 1:1000, 1:5000, 1:2000, and 1:2000 dilution, respectively. Antibodies against total p38, ERK, JNK, and ASK1 (all from Cell Signaling, Danvers, MA) were used at 1:2000, 1:5000, 1:2000, and 1:2000 dilution, respectively. Blots were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression (1:10000 dilution, Chemicon International, Temecula, CA).

Factor Xa generation

To assess TF activity factor Xa generation on the surface of VSMC and HAEC was analyzed using a colorimetric assay (American Diagnostica Inc, ACTICHROME). TF/FVIIa complex converted human factor X to factor Xa, which was measured by its ability to metabolize a chromogenic substrate. The absorbance of the reaction mixture was measured at 405 nm and values displayed after substraction of the background value. To confirm TF dependency of factor Xa generation in VSMC, cells were incubated with an inhibitory anti-TF antibody (American Diagnostica, No. 4509; 1:200) 30 minutes prior addition of exogenous factor VIIa.

For analysis of factor Xa generation in mouse carotid arteries, occluded right

carotid arteries from treated and control mice were homogenized in 50 µL lysis buffer (0.1% Triton-X, 100 mmol Nacl, 50 mmol Tris-Hcl, pH 7.4). 25 µg of tissue lysates were used for measurements. TF dependency of factor Xa generation in tissue lysates was confirmed by incubation with an inhibitory mouse anti-TF antibody (American Diagnostica; No. 4515; 10 mg/ml) 30 minutes prior addition of exogenous factor VIIa.

Real Time PCR

RNA was extracted from VSMC, HAEC and mouse aorta using TRIzol Reagent (Invitrogen, Carlsbad, CA) and reverse transcribed using Ready-To-Go You-Prime First-Strand Beads (Amersham, Buckinghamshire, UK). For real-time PCR analysis the following primers were used: for full length human TF (*F3*): sense 5'-TCCCCAGAGTTCACACCTTACC–3', antisense 5'–CCTTTCTCCTGGCCCATACAC–3'; for human ribosomal L28: sense 5'-GCATCTGCAATGGATGGT-3', antisense 5′-TGTTCTTGCGGATCATGTGT-3′; for murine full length tissue factor: sense: 5'-CAATGAATTCTCGATTGATGTGG-3', antisense: 5'-GGAGGATGATAAAGATGGTGGC-3'; for murine tissue factor pathway inhibitor: sense: 5'-ACTGTGTGTCTGTTGCTTAGCC-3', antisense: 5'-GTTCTCGTTCCCTTCACATCCC3'; and for murine ribosomal S12: sense: 5'-GAAGCTGCCAAAGCCTTAGA-3', antisense: 5'-AACTGCAACC-AACCACCTTC-3'. The amplification program consisted of 1 cycle at 95°C for 10 minutes followed by 35 cycles with a denaturing phase at 95°C for 30 seconds, an annealing phase at 60°C for 1 minute, and an elongation phase at 72° for 1 minute. Melting curve analysis confirmed the accuracy of the amplicon, and PCR products were analyzed on an ethidium bromide stained 1% agarose gel. In each real-time PCR run for human TF and L28, a standard curve generated from serial dilutions of purified amplicons was

included. For murine TF and S12 the ΔΔCt threshold cycle method was used.

Nuclear Extracts and NFκB activity

For measurement of NFkB activity, VSMC were treated with ALA (30 μM) or the vehicle for 24 hours. Nuclear extracts were obtained from VSMC or mouse aorta using a nuclear extraction kit (Active Motif, Carlsbad, USA). Total protein (20 μg) was loaded in each well, and NFκB activity was measured using a TransAM NFκB p65 kit (Active Motif).

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Laminin Receptor Activation Inhibits Endothelial Tissue Factor Expression

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Abstract

Background. Tissue factor (TF) is an important trigger of arterial thrombosis. The green tea catechin epigallocatechin-3-gallate (EGCG) is a ligand of the 67 kDa laminin receptor (67LR) and exhibits cardioprotective effects. This study investigates whether 67LR regulates TF expression in human endothelial cells.

Methods and Results. Immunofluorescence demonstrated that human aortic endothelial cells expressed 67LR. Cells grown on laminin expressed 35% less TF in response to TNF-alpha (TNF-α) than those grown on fibronectin (n=6; $p<0.001$). EGCG (1-30 μM) inhibited TNF-α and histamine induced endothelial TF expression and activity in a concentration dependent manner resulting in 87% reduction of TF expression (n=5; $p<0.001$); in contrast, expression of tissue factor pathway inhibitor was not affected (n=4; p=NS). In vivo administration of EGCG (30 mg/kg/d) inhibited TF activity in carotid arteries of C57BL6 mice. Real-time PCR and promoter studies revealed that EGCG decreased TF expression at the transcriptional level and impaired activation of the mitogen activated protein (MAP) kinase JNK 1/2, but not ERK or p38. Similarly, the JNK 1/2 inhibitor SP600125 (1 μM) impaired TF promoter activity (n=4; $p<0.001$) and protein expression (n=4; $p<0.001$). 67LR blocking antibodies blunted the inhibitory effect of EGCG on both TF protein expression and JNK activation. In contrast, vascular cell adhesion molecule 1 (VCAM-1) was not affected by laminin nor EGCG, and its expression was not regulated by JNK. EGCG did not affect TNF-α stimulated NFkB activation.

Conclusions. Laminin receptor activation inhibits endothelial TF expression by impairing JNK phosphorylation. Thus, 67LR may be a potential target for the development of novel anti-thrombotic therapies.

Introduction

Tissue factor (TF) plays an important role in initiating coagulation [1]. Inflammatory mediators such as TNF-alpha (TNF-α) or histamine are potent inducers of TF expression in vascular cells [2]. Elevated levels of TF are detected in atherosclerotic plaques, and an involvement of TF in drug-eluting stent thrombosis has been discussed as well [3,4]. These findings suggest that modulation of TF expression in vascular cells may offer a strategy for the treatment and prevention of arterial thrombosis.

The extracellular matrix (ECM) plays a pivotal role in vascular homeostasis, since it maintains vascular integrity by regulating proliferation, migration, and morphogenesis of endothelial cells [5,6]. Indeed, the basement membrane forms a scaffold around the endothelial tube, and interactions between endothelial cells and constituents of the basement membrane are essential for vessel wall integrity [7]. The non-collagenous glycoprotein laminin is a cross shaped heterotrimer of α, β, and γ subunits and a major component of the basement membrane [7]. The membrane bound 67 kDa-laminin receptor (67LR) is a non-integrin cell surface receptor directly interacting with the β_1 subunit of laminin [8]. Besides its role in cell adherence to the extracellular matrix, there is growing evidence that 67LR activation induces functional changes within cells [8]. 67LR has indeed been implicated in shear stress induced endothelial nitric oxide synthase (eNOS) activation, since its blockade abolished shear induced eNOS phosphorylation [9]. Despite such observations, the role of 67LR in endothelial activation remains incompletely understood, and possible implications for arterial thrombosis have not been investigated.

Recently, 67LR has been identified as a cell surface receptor for the green tea polyphenol epigallocatechin-3-gallate (EGCG) [10]. EGCG is the most abundant

polyphenol found in green tea and constitutes 30% of its dry mass. Experimental studies revealed cardioprotective properties of ECGC consisting in anti-inflammatory, anti-oxidant, and anti-atherogenic effects [11-16]; moreover, large epidemiological trials demonstrated that green tea consumption is inversely associated with cardiovascular mortality [17-19].

The present study investigates whether 67LR modulates TF expression in human aortic endothelial cells.

Methods

Cell culture

Human aortic endothelial cells (HAEC; Clonetics, Allschwil, Switzerland), human umbilical venous endothelial cells (HUVEC; Clonetics) and human aortic vascular smooth muscle cells (HASMC, Clonetics) were cultured on fibronectin as described [20]. Cells were rendered quiescent in medium supplemented with 0.5% fetal bovine serum for 24 hours before stimulation with 5 ng/mL TNF-α (R&D Systems, Minneapolis, MN) or 10 μM histamine (Sigma, St. Louis, MO). Cells were pretreated with EGCG (Sigma) for 60 minutes. For studies of VCAM-1 and TF protein expression HAEC were stimulated for 5 hours. To block the mitogen activated protein (MAP) kinase c-Jun terminal NH_2 kinase 1/2 (JNK 1/2), HAEC were treated with SP600125 (Calbiochem, Lucerne, Switzerland) for 60 minutes before stimulation. Cytotoxicity was assessed using a colorimetric assay for detection of lactate dehydrogenase release (Roche, Basel, Switzerland)**.** Where mentioned, dishes were coated with laminin (25 μg/mL; Sigma). To compensate for the slower growth rates of HAEC on laminin as compared to fibronectin, a higher cell number was seeded on the dishes; the two groups exhibited similar confluency at the time of the experiments. To study the role of 67LR, cells were pretreated for 60 minutes with either a monoclonal (MLuC5, 1:1'000) or a polyclonal (ab711, 1:1'000) anti-human 67LR blocking antibody or the appropriate isotype antibodies (all from Abcam, Cambridge, UK).

Immunofluorescence microscopy

HAEC were grown on LabTek chamber slides (Nunc, Rochester, MN), washed

three times with PBS, and fixed with 4% paraformaldehyde for 15 min. Cells were washed twice with PBS and incubated with anti-67LR (MLuC5) antibody for 1 hour (1:1'000), followed by incubation with Alexa-488 conjugated secondary antibody (Invitrogen, Carlsbad, CA) for 1 hour (1:2'000). Antibodies were diluted in PBS containing 1% bovine serum albumin. After washing with PBS, cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories Inc., Burlingame, CA), mounted on chamber slides, and examined by a fluorescence microscope (Olympus, DP40, Tokyo; Japan).

Western blot

Protein expression was determined by Western blot analysis. Antibodies against human TF, tissue factor pathway inhibitor (TFPI) (both from American Diagnostica, Stamford, CT), and vascular cell adhesion molecule-1 (VCAM-1; R&D Systems, Minneapolis, MN) were used at 1:2500 dilution. Antibodies against phosphorylated p38 MAP kinase (p38), p44/42 MAP kinase (extracellular signal regulated kinase [ERK]), and c-Jun NH2 terminal kinase (JNK1/2; all from Cell Signaling, Danvers, MA) were used at 1:1000, 1:5000, and 1:1000 dilution, respectively. Antibodies against total p38, ERK, and JNK1/2 (all from Cell Signaling) were used at 1:2000, 1:5000, and 1:1000 dilution, respectively. IkB-α expression pattern was assessed by anti-human IkB-α antibody (1:5000; Cell Signaling). All blots were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression (1:10'000 dilution, Chemicon International, Temecula, CA). The quantification of protein was performed by densitometric analysis using Scion Image Software (Scion Corp., Frederick, MD).

TF surface activity

TF surface activity was analyzed on HAEC after 5 hours of stimulation with TNF-α in the presence or absence of EGCG (30 μM)_using a colorimetric assay (American Diagnostica). Briefly, TF/FVIIa complex converted human factor X to factor Xa, which was measured by its ability to metabolize a chromogenic substrate. The absorbance of the reaction mixture was measured at 405 nm, and TF activity was determined from a standard curve performed with lipidated human TF.

In vivo study

Analysis of TF activity was performed in 10 week old male C57BL6 mice (Jackson Laboratories, Bar Harbor, ME) weighing an average of 26 g and fed a normal chow diet (KLIBA NAFAG, Kaiseraugst, Switzerland). EGCG was dissolved in PBS (pH 7.4) and administrated i.p. at a dose of 30 mg/kg/d for 7 days. Age, sex, and weight matched controls received an equal volume of vehicle. Mice were then euthanized and the left common carotid artery harvested for analysis of TF activity using a colorimetric assay (American Diagnostica).

Real-time PCR

RNA was extracted from HAEC using TRIzol Reagent (Invitrogen, Carlsbad, CA) and real-time PCR performed as described [20]. The following primers were used: for full length human TF (*F3*): sense 5'-TCCCCAGAGTTCACACCTTACC–3' (bases 508-529 of *F3* cDNA; NCBI no. NM 001993), antisense 5'–CCTTTCTCCTGGCCCATACAC–3'(bases 843-863 of *F3* cDNA; NCBI no. NM 001993); for human ribosomal L28: sense 5'-GCATCTGCAATGGATGGT-3', antisense 5'–CCTTTCTCCTG-GCCCATACAC-3'. Melting curve analysis was performed after amplification to verify the accuracy of the amplicon, and PCR products were analyzed on an ethidium bromide stained 1% agarose gel. In each

real-time PCR run for TF and L28, a standard curve generated from serial dilutions of purified amplicons was included.

TF promoter activity

TF promoter activity was measured as described [21]. Briefly, the minimal TF promoter (-227 bp to +121 bp) was cloned upstream of the firefly luciferase reporter gene using a pGL2-Basic plasmid (Promega, Madison, WI). For transduction, the adenoviral vector Ad5/hTF/Luc was added to HAEC at an multiplicity of infection of 100 plaque forming units/cell for 1 hour and then removed. The adenoviral vector VQAd Empty was used as a negative control. HAEC were kept in growth medium for 24 hours and then serum starved for 24 hours prior to TNF-α stimulation for 1 hour with or without EGCG (30 μM). Firefly luciferase activity was determined in cell lysates using a luminometer (Berthold Technologies, Bad Wildbad, Germany) and normalized by determing protein concentration in the lysates.

Transfection with siRNA

HAEC were transfected with siRNA (15 nM) against JNK 1 (5'-AAAGAAUGUCCUACCUUCUTT-3'), JNK 2 (5'-AGAAGGUAGGA-CAUUCUUUTT-3') or scrambled RNA (all from Sigma) using the N-TER™ Nanoparticle siRNA Transfection System (Sigma) for 4 hours. After transfection, HAEC were grown for 12 hours and then serum starved for 24 hours prior to the experiment.

Nuclear extraction and NFkB activity assay

For NFκB activity measurements, HAEC were stimulated with TNF-α for 30 minutes in the presence or absence of EGCG (30 μM). Nuclear extracts were obtained from HAEC using a nuclear extraction kit (Active Motif, Carlsbad, USA).

Nuclear protein (20 µg) was loaded in each well, and NFκB activity was measured using a TransAM NFkB p65 kit (Active Motif) according to manufacturer's recommendations.

Statistics

Data are presented as mean±SEM. Statistical analysis was performed using two tailed unpaired Student´s t-test or one-way ANOVA as appropriate. A value of $p<0.05$ was considered significant. All results are representative of at least four independent experiments.

Results

Laminin inhibits TF protein expression

Immunofluorescence microscopy revealed that 67LR was abundantly expressed on the surface of HAEC (Figure 1A). Western blot analysis demonstrated that TNF-α (5ng/mL) induced TF expression was 35% lower in cells growing on laminin as compared to those growing on fibronectin (n=4; $p<0.001$; Figure 1B).

67LR inhibits TF protein expression

EGCG (1-30 μM) inhibited TNF-α (5 ng/mL) induced TF expression in a concentration dependent manner (n=5; *$p<0.001$; figure 2A); the maximal effect occurred at 30 μM and decreased TF expression by 87% as compared to TNF-α alone. Similarly, EGCG (30 μM) inhibited histamine (10 μM) induced TF expression by 84% as compared to histamine alone (n=5; *$p<0.001$; figure 2B). EGCG did not affect expression of TFPI (n=4; p=NS; supplemental figure 1). The effect of EGCG on TF protein expression was paralleled by a reduced TF surface activity (n=4; *$p<0.001$; figure 2C). Further the effect of EGCG on TF activity was assessed in carotid artery lysates of C57BL6 mice. EGCG administrated for 7 days (30 mg/kg/d) reduced TF activity by 46.6±17% as compared to control animals (n=5; $p<0.05$; figure 2D). In line with this observation, EGCG also impaired TNF-α induced TF expression in cultured HASMC and HUVEC (n=4; $p<0.01$; supplemental figure 2 and 3). Pretreatment of HAEC with the monoclonal 67LR blocking antibody MLuC5 blunted the inhibitory effect of EGCG on TF protein expression as compared to cells treated with the isotype control (n=4; *$p<0.001$ vs TNF-α alone; figure 2E). A similar effect was observed using a polyclonal 67LR antibody (n=4; *$p<0.001$; data not shown). LDH release was not altered by any of the EGCG concentrations used (n=4; p=NS;

data not shown).

67LR inhibits TF mRNA expression and promoter activity but not TNF-α induced NFkB activation

TNF-α induced TF mRNA expression within 2 hours of stimulation as determined by real-time PCR analysis (n=5; *p<0.0001; Figure 3A). EGCG (30 μM) impaired TNF-α induced TF mRNA expression by 74% as compared to TNF-α alone (n=5; *p<0.001; Figure 3A). EGCG (30 μM) did not alter TNF-α stimulated IkB-α degradation nor TNF-α induced nuclear translocation and DNA binding activity of the NF kappa B subunit p65 (n=4; p=NS, figure 3B and 3C). To assess whether the effect on TF mRNA was mediated via promoter inhibition, the impact of EGCG on TF promoter activity was analyzed after adenoviral transduction of endothelial cells with firefly luciferase under control of the human minimal TF promoter (-221 bp to +121 bp). TNF-α enhanced TF promoter activity by 2.2 fold as compared to control conditions (n=4; *p<0.001; Figure 3D); while EGCG (30 μM) inhibited this effect by 51% (n=4; *p<0.001; Figure 3D).

67LR inhibits JNK1/2 phosphorylation

To determine whether 67LR inhibited MAP kinases, phosphorylation of these mediators was assessed at different time points after TNF-α stimulation. TNF-α (5 ng/mL) transiently activated JNK1/2, ERK, and p38; maximal activation was observed after 15 minutes and returned to basal levels within 60 minutes (Figure 4A). EGCG inhibited phosphorylation of JNK by 46% as compared to TNF-α alone (n=4; *p<0.01; Figure 4B), while phosphorylation of ERK and p38 was not altered (n=4; p=NS; Figure 4B). Total expression of JNK 1/2, ERK, and p38 remained unaffected at all time points examined (Figure 4A). Targeting of JNK1 and JNK2 with specific

siRNA impaired TNF-α induced TF expression by 33% and 60%, respectively as compared to TNF-α alone (n=4; $p<0.05$ for JNK1 vs TNF-α alone and $p<0.01$ for JNK2 vs TNF-α alone; figure 5A). Inhibition of JNK 1 and 2 phosphorylation with SP600125 mimicked the effect of siRNA on TF protein expression (n=4; *$p<0.001$; Figure 5B) and abolished TNF-α induced TF promoter activation (n=4; *$p<0.001$; Figure 5C). The inhibitory effect of EGCG on JNK phosphorylation was prevented by pretreatment with the 67LR blocking antibody MLuC5 (n=3; $p<0.01$; Figure 5D).

Neither 67LR nor JNK 1/2 regulate VCAM-1 expression

Western blot analysis revealed that TNF-α induced VCAM-1 expression (n=4; $p<0.001$; Figure 5A) in HAEC. Neither 67LR activation by laminin or EGCG (1-30 μM) nor JNK inhibition by SP600125 (n=4; *p*=NS; Figure 6A, 6B, and 6C) impaired TNF-α induced VCAM-1 expression. Similar to the result obtained in HAEC with EGCG in HUVEC (n=4; p=NS; supplemental figure 4).

Discussion

This study demonstrates that activation of the 67LR by laminin or EGCG inhibits TNF-α induced TF expression in endothelial cells. Moreover, administration of EGCG in C57BL6 mice diminishes TF activity in carotid artery lysates. This effect is mediated at the transcriptional level via reduced phosphorylation of the JNK isoforms p46 and p54 (JNK1 and JNK2) and impaired TF promoter activation. In contrast, 67LR does not affect the JNK 1/2 independent expression of VCAM-1 in these cells.

Endothelial cells line the vascular lumen while their abluminal surface interacts with the basal membrane. The laminin 10 isoform is the main component of the basal membrane in mature vessels, separating endothelial cells from interstitial collagens [7]. It is notable that the laminin most widely used for in vitro studies was isolated from mouse Engelberth-Holm-Swarm tumors corresponding to the laminin 1 isotype [7]. This form of laminin, however, does not occur in the endothelial cell basement membrane and therefore might constitute a limitation for previous studies. Therefore, the effect of laminin on TNF-α induced TF expression was analysed in this study using commercially available human laminin and mainly containing the laminin 10 isoform.

Separation of endothelial cells from interstitial collagens plays an important role in maintaining vessel wall integrity, since laminin and interstitial collagen modulate endothelial function in a differential manner. Indeed, collagen activates endothelial Src kinase as well as the GTPase Rho while suppressing the activity of the GTPase Rac, leading to disruption of intercellular junctions, cell proliferation, tube formation, and initiation of angiogenesis [5,6]. In contrast, laminin antagonizes these effects [5,6]. In line with these findings, cells growing on laminin proliferate slower than those on fibronectin. The present study demonstrates that endothelial cells

growing on laminin express lower levels of TF upon stimulation with TNF-α as compared to those on fibronectin. This observation is consistent with the interpretation that basal membrane associated laminin preserves vascular integrity by reducing endothelial cell activation.

The interaction between 67LR and the laminin subunit β1 is an essential component of cellular adhesion to the basement membrane [8]. The polyphenol EGCG has been characterized as a ligand for 67LR. Experiments performed in human lung cancer cells demonstrate that EGCG mediated growth inhibition is 67LR dependent and that EGCG binds to the 67LR with a K_d value of 39.9 nM [10]. In endothelial cells, 67LR is involved in shear stress induced endothelial nitric oxide synthase activation, indicating that this receptor has functional properties beyond its known role in cell adherence [9]. The present study demonstrates that 67LR is abundantly expressed on the surface of human aortic endothelial cells and that activation of 67LR by laminin or EGCG inhibits TF protein expression and activity.

TF expression is a major trigger of the thrombotic response, and high levels of TF contribute to thrombus formation following plaque rupture or erosion [4]. The proinflammatory cytokine TNF-α [22] and histamine have been identified as potent inducers of TF expression [2]. Similar to laminin, EGCG inhibited TNF-α and histamine induced TF expression in a concentration dependent manner. The MAP-kinases JNK1/2, p38, and ERK regulate TF expression at the transcriptional level in response to both agonists [23]. 67LR activation by EGCG resulted in decreased phosphorylation of the MAP kinase JNK1/2 and decreased expression of TF. Conversely, 67LR blockade abolished the inhibitory effects of EGCG on JNK1/2 activation and TF expression. Finally, specific targeting of JNK1/2 with siRNA or the pharmacological inhibitor SP600125 reduced TNF-α induced TF promoter activity, RNA and protein expression. Hence, EGCG inhibits TF expression by decreasing

JNK activation.

An involvement of MAP kinases in laminin signalling has been described in tumor cells [24]. To confirm the role of JNK1/2 in mediating the effects of 67LR activation on endothelial TF, TNF-α induced VCAM-1 expression was analyzed, since this protein, is regulated independent of JNK1/2 in this cell type [25]. Neither laminin nor EGCG affected endothelial VCAM-1 expression, confirming that 67LR activation specifically inhibits JNK1/2. It is of note that some previous studies described a role for JNK1/2 in VCAM-1 induction in vascular cells [26;27]. However, it should be considered that different cell types, different experimental protocols, and different SP600125 concentrations may account for these discrepancies. TNF-α induced VCAM-1 expression depends on NFkB activation in endothelial cells [28]. EGCG did not affect IkB-α degradation nor NFkB activation after TNF-α stimulation in HAEC. This result strongly suggests that EGCG inhibits TF expression via a transcription factor other than NFkB and located downstream of JNK1/2, such as AP-1, which is known to regulate TF promoter activation [29].

It is of clinical relevance that the inhibition of TF mRNA and protein expression reached a significant level when EGCG was applied at concentrations as low as 1 and 3 μM. Such levels of EGCG have been measured in human plasma following the oral intake of green tea extract or purified EGCG [30,31]. Thus, the inhibitory effect of 67LR activation by EGCG occurred at clinically relevant concentrations and might account at least in part for the cardioprotective effects of green tea consumption.

In summary, this study demonstrates that 67LR activation by laminin or the polyphenol EGCG inhibits endothelial TF expression and activity. Hence, it describes a new mechanism by which the basement membrane modulates the hemostatic balance in intact vessels and identifies 67LR as a potential target for the development of novel anti-thrombotic therapies.

Acknowledgements

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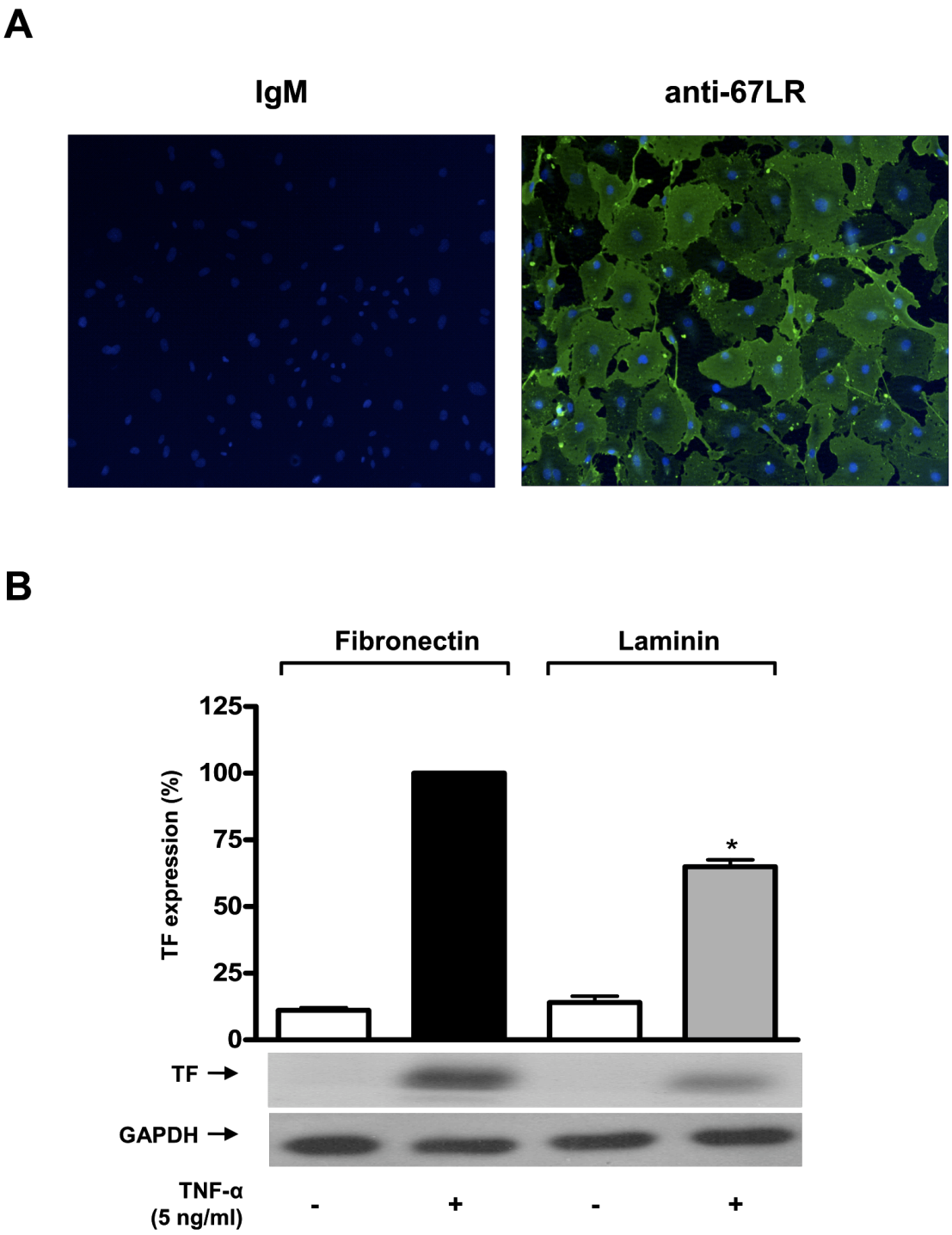

A
IgM
anti-67LR
B
Fibronectin
Laminin
125
100
75
50
25
0
TF expression (%)
*
TF
GAPDH
TNF-α
(5 ng/ml)
-
+
-
+

Figure 1

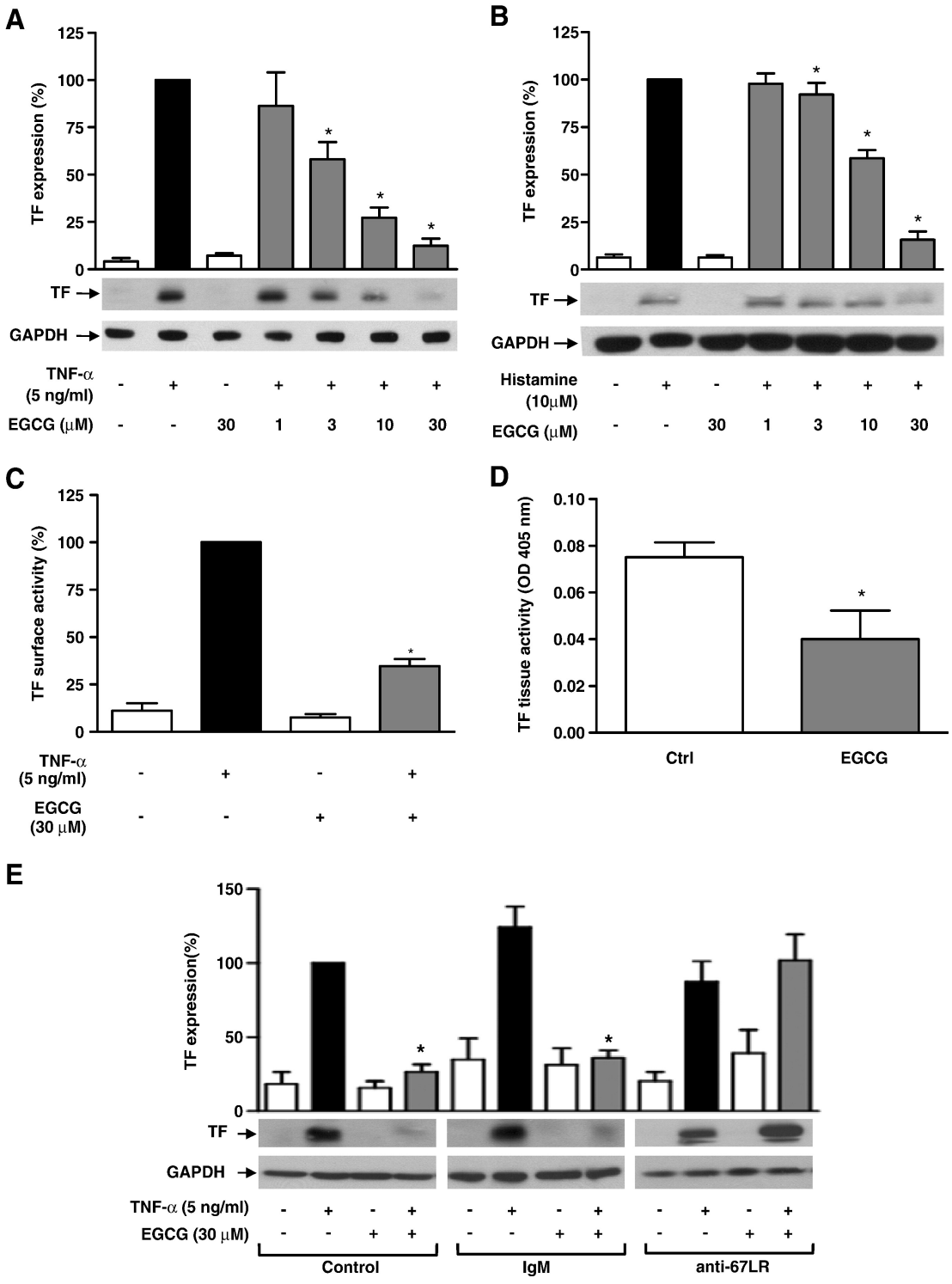

A
TF expression (%)
125
100
75
50
25
0
TF
GAPDH
TNF-α (5 ng/ml)
EGCG (μM)
- + - + + + +
- - 30 1 3 10 30
B
TF expression (%)
TF
GAPDH
Histamine (10μM)
EGCG (μM)
- + - + + + +
- - 30 1 3 10 30
C
TF surface activity (%)
TNF-α (5 ng/ml)
EGCG (30 μM)
- + - +
- - + +
D
TF tissue activity (OD 405 nm)
0.10
0.08
0.06
0.04
0.02
0.00
Ctrl
EGCG
E
TF expression(%)
150
100
50
0
TF
GAPDH
TNF-α (5 ng/ml)
EGCG (30 μM)
- + - + - + - + - + - +
- - + + - - + + - - + +
Control
IgM
anti-67LR

Figure 2

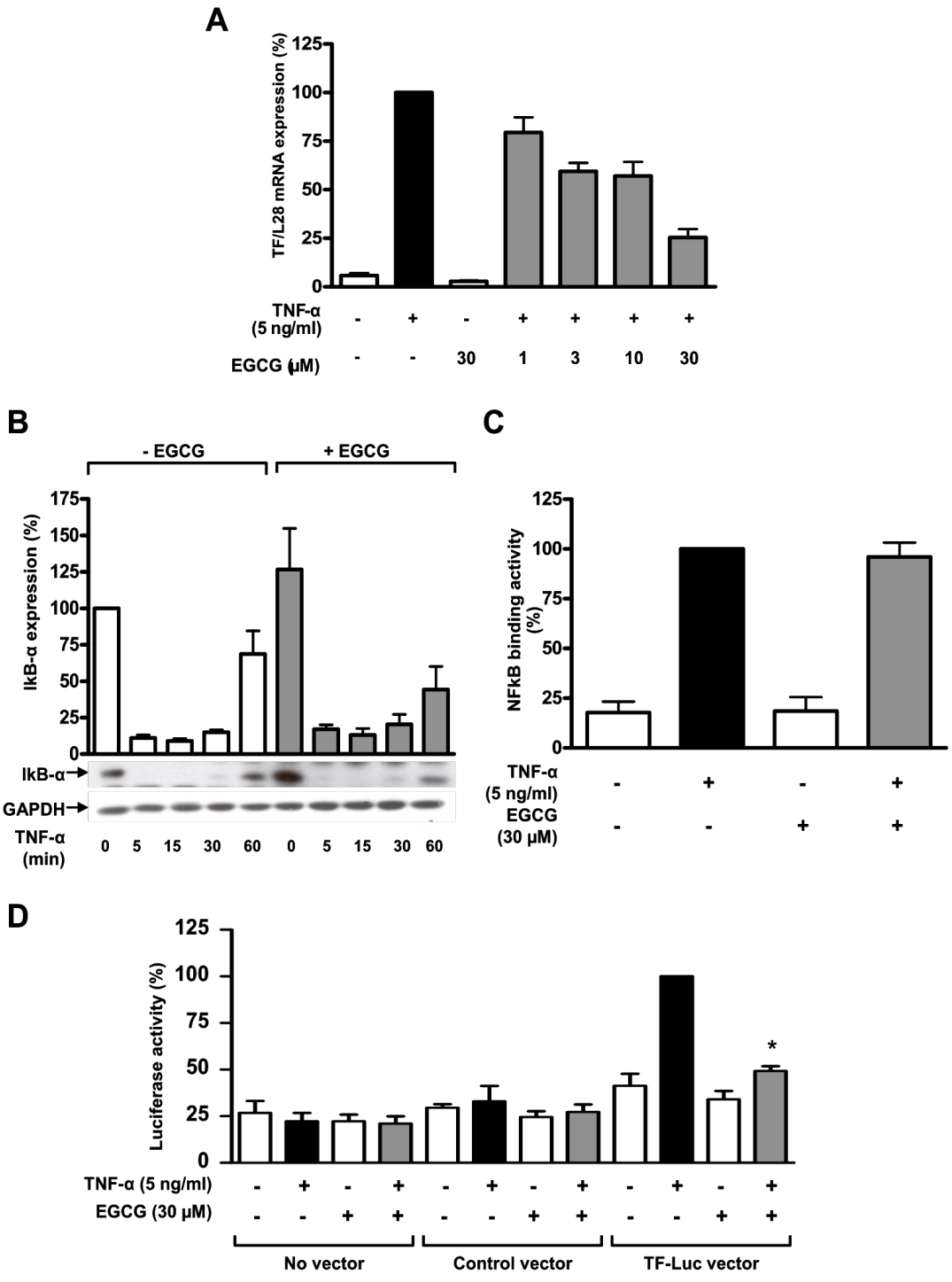
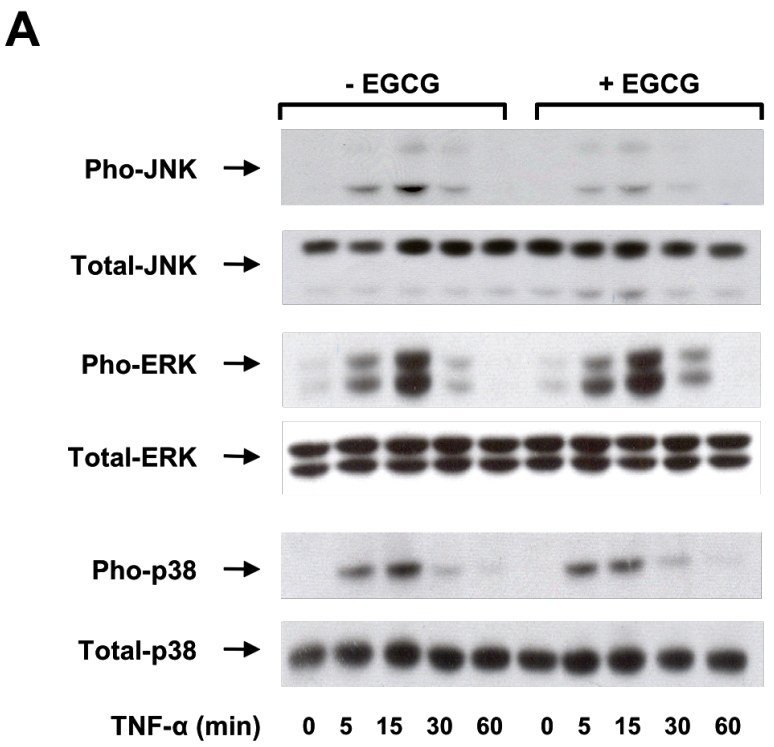

A
TF/L28 mRNA expression (%)
125
100
75
50
25
0
TNF-α (5 ng/ml) - + - + + + +
EGCG (μM) - - 30 1 3 10 30
B
- EGCG
+ EGCG
IkB-α expression (%)
175
150
125
100
75
50
25
0
IkB-α
GAPDH
TNF-α (min) 0 5 15 30 60 0 5 15 30 60
C
NFkB binding activity (%)
125
100
75
50
25
0
TNF-α (5 ng/ml) - + - +
EGCG (30 μM) - - + +
D
Luciferase activity (%)
125
100
75
50
25
0
*
TNF-α (5 ng/ml) - + - + - + - + - + - +
EGCG (30 μM) - - + + - - + + - - + +
No vector
Control vector
TF-Luc vector

Figure 3


A
- EGCG
+ EGCG
Pho-JNK
Total-JNK
Pho-ERK
Total-ERK
Pho-p38
Total-p38
TNF-α (min)
0 5 15 30 60 0 5 15 30 60

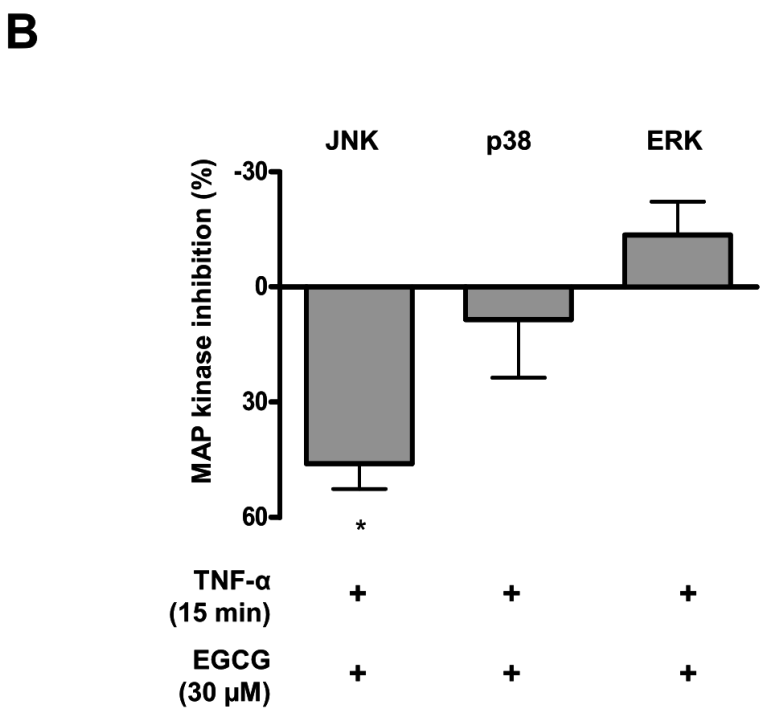

B
JNK
p38
ERK
MAP kinase inhibition (%)
-30
0
30
60
*
TNF-α
(15 min)
EGCG
(30 μM)
+
+
+
+
+
+

Figure 4

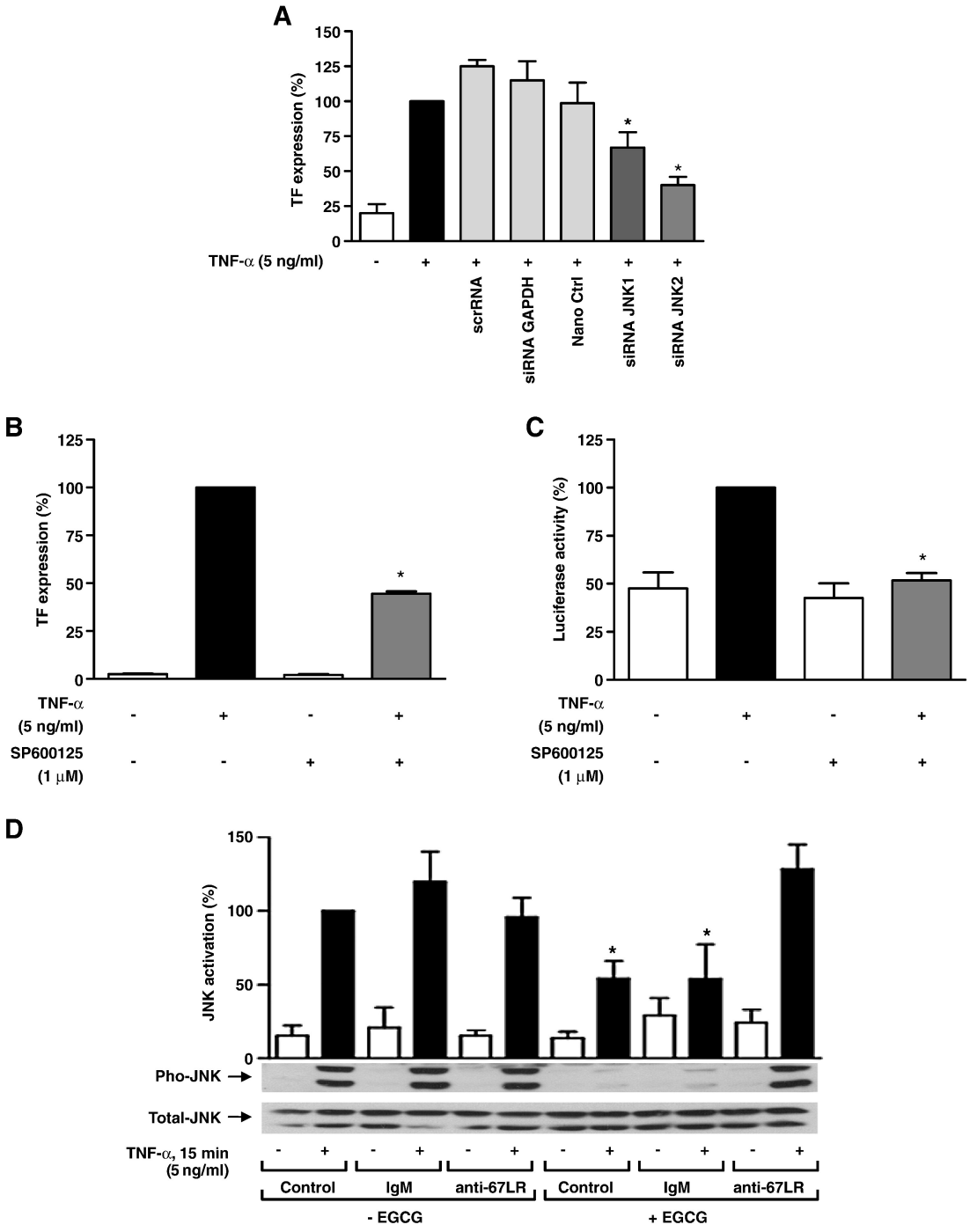

A
TF expression (%)
150
125
100
75
50
25
0
TNF-α (5 ng/ml)
-
+
scrRNA
siRNA GAPDH
Nano Ctrl
siRNA JNK1
siRNA JNK2
B
TF expression (%)
TNF-α (5 ng/ml)
SP600125 (1 μM)
C
Luciferase activity (%)
TNF-α (5 ng/ml)
SP600125 (1 μM)
D
JNK activation (%)
Pho-JNK
Total-JNK
TNF-α, 15 min (5 ng/ml)
Control
IgM
anti-67LR
- EGCG
+ EGCG

Figure 5

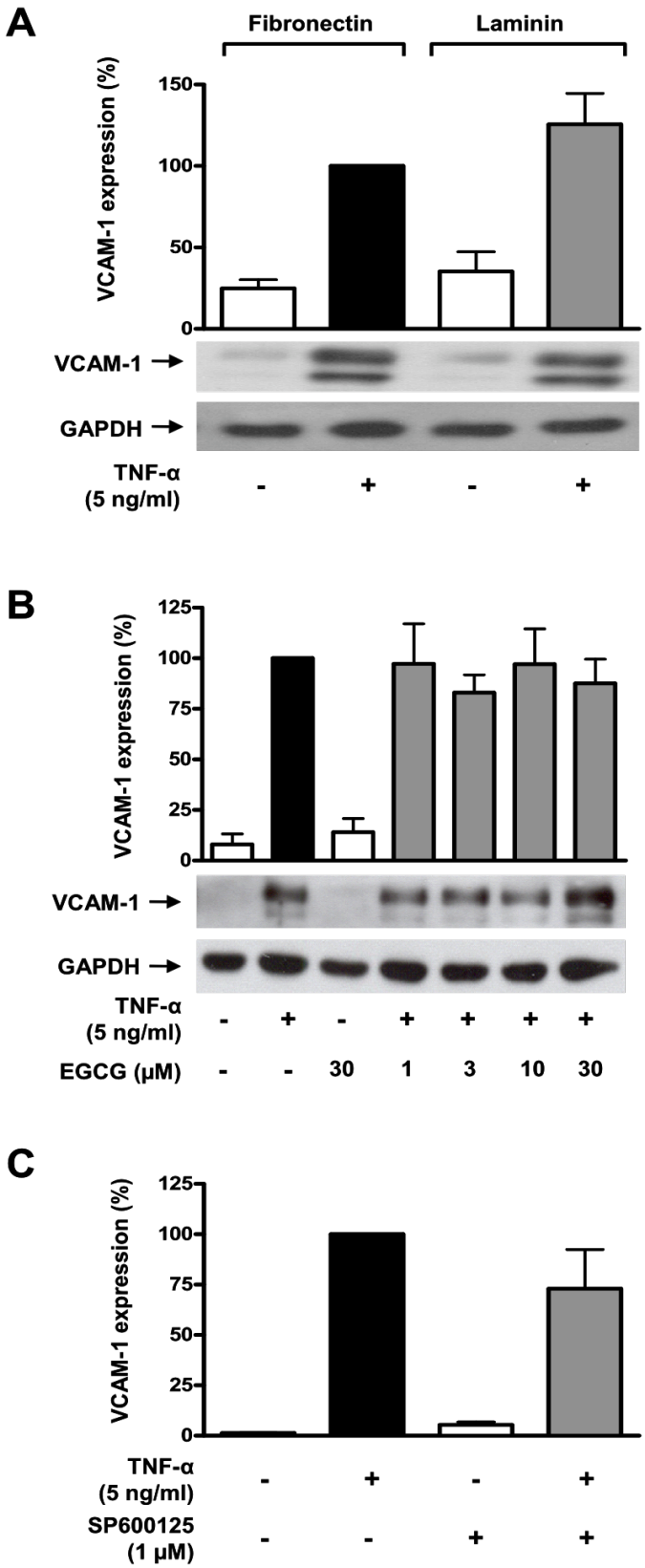

A
Fibronectin
Laminin
VCAM-1 expression (%)
150
100
50
0
VCAM-1
GAPDH
TNF-α
(5 ng/ml)
- + - +
B
VCAM-1 expression (%)
125
100
75
50
25
0
VCAM-1
GAPDH
TNF-α
(5 ng/ml)
- + - + + + +
EGCG (μM)
- - 30 1 3 10 30
C
VCAM-1 expression (%)
125
100
75
50
25
0
TNF-α
(5 ng/ml)
- + - +
SP600125
(1 μM)
- - + +

Figure 6

Figure Legends

Figure 1: Laminin inhibits TF protein expression

A. 67LR is abundantly expressed on the surface of HAEC. 20x magnification. **B**. TNF-α induced TF expression is inhibited when cells are grown on laminin as compared to fibronectin. *p<0.001.

Figure 2: 67LR inhibits TF protein expression

EGCG inhibits TNF-α (**A**) and histamine (**B**) induced TF protein expression in HAEC. *p<0.001 vs TNF-α and histamine alone. **C**. EGCG decreases TNF-α induced TF surface activity. *p<0.001 vs TNF-α alone. **D**. In C57BL6 mice EGCG reduces TF activity. *p<0.05 vs control. **E**. Treatment with a 67LR blocking antibody (MLUC5) abrogates the inhibitory effect of EGCG on TF expression. *p<0.01 vs TNF-α alone. All blots are normalized to GAPDH.

Figure 3: 67LR inhibits TF promoter and TF mRNA expression

A. Real-time PCR analysis demonstrates that EGCG impairs TF mRNA expression in HAEC. Values are normalized to L28 expression. *p<0.0001 vs TNF-α alone. **B**. Luciferase assay shows that EGCG inhibits TNF-α induced TF promoter activity. Values are normalized to total protein concentration. *p<0.001 vs TNF-α alone.

Figure 4: EGCG impairs JNK1/2 phosphorylation

A. TNF-α induces a transient phosphorylation of the MAP-kinases JNK, ERK, and p38. EGCG inhibits phosphorylation of JNK1/2, but not ERK and p38. No change in total expression of JNK, ERK, and p38 is observed. **B**. EGCG inhibits activation of

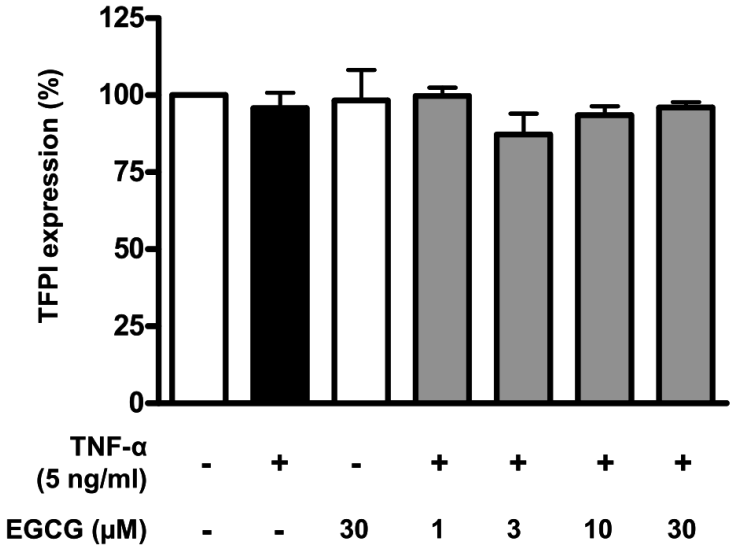
JNK, but not ERK and p38. *p<0.01 vs TNF-α alone.

Figure 5: 67LR activation mediates impairment of JNK1/2 activation by EGCG

A. Transfection with siRNA against JNK1 and JNK2 mRNA inhibits TNF-α induced TF expression. *p<0.05 vs TNF-α alone. SP600125, a specific JNK inhibitor, impairs TF protein expression (**B**) and TF promoter activation (**C**). *p<0.001 vs TNF-α alone. **D**. Treatment with a 67LR blocking antibody blunts the inhibitory effect of EGCG on JNK activation. *p<0.001 vs TNF-α alone.

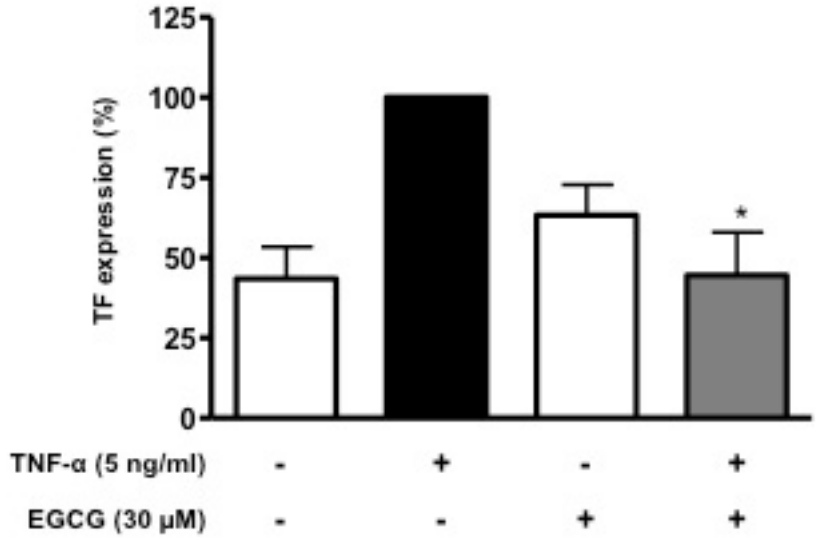
Figure 6: Neither 67LR nor JNK regulate VCAM-1 expression

A. Laminin does not alter TNF-α induced VCAM-1 expression in HAEC as compared to fibronection. p=NS. **B**. EGCG has no effect on TNF-α induced VCAM-1 expression. p=NS vs TNF-α alone. **C**. JNK inhibition with SP600125 does not affect TNF-α induced VCAM-1 expression. p=NS vs TNF-α alone. All blots are normalized to GAPDH.



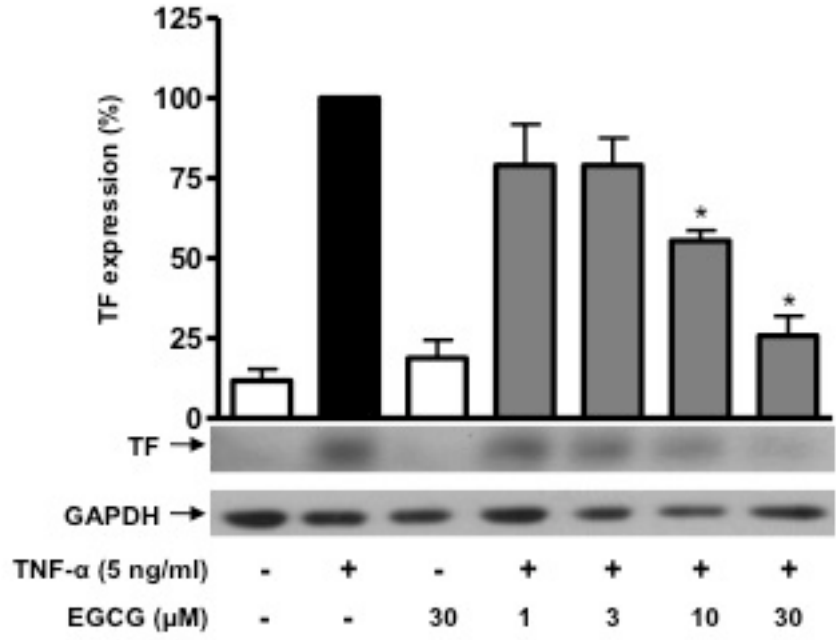
Supplemental figure 1. EGCG does not affect TFPI expression.

Incubation with EGCG does not alter endothelial TFPI expression. *p*=NS vs unstimulated control and *p*=NS vs TNF-α alone.



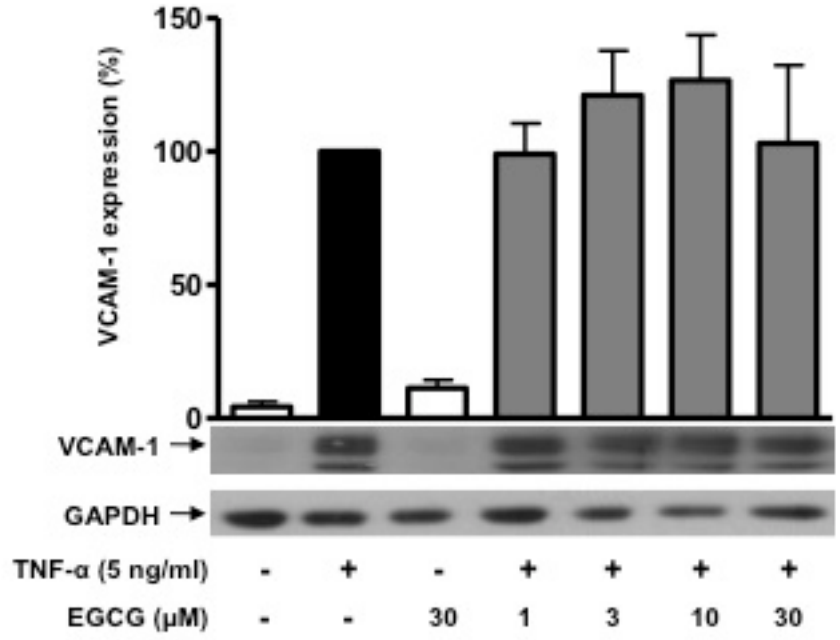
Supplemental figure 2: EGCG inhibits TF expression in HASMC.

ECGC inhibits TNF-α induced TF expression in HASMC. *p<0.01 vs TNF-α alone.



Supplemental figure 3: EGCG impairs TF expression in HUVEC.

In HUVEC ECGC impairs TNF- α induced TF expression. *p<0.01 vs TNF-α alone.



Supplemental figure 4: EGCG does not alter VCAM-1 expression in HUVEC.

EGCG has no effect on TNF-α induced VCAM-1 expression in HUVEC. *p*=NS vs TNF-α alone.

Berberine, a Natural Lipid-Lowering Drug, Exerts Prothrombotic Effects on Vascular Cells

Role of Tissue Factor mRNA Stabilization

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* these authors contributed equally to this work

Abstract

Aims. Berberine (BBR) is a novel natural hypolipidemic agent. This study investigates whether BBR, similar to statins, exerts pleiotropic effects on endothelial tissue factor (TF) expression.

Methods and Results. BBR enhanced tumor necrosis factor-α (TNF-α) and thrombin induced TF expression in human endothelial cells by 3.5-fold. These effects were paralleled by an enhanced TF surface activity. In contrast, expression of TF pathway inhibitor was impaired. BBR enhanced TNF-α induced TF mRNA expression; however, TF promoter activity was inhibited. Activation ERK and p38 remained unaffected, while c-Jun terminal NH_2 kinase was inhibited. BBR reduced TF mRNA degradation rates, prolonging its half-life from 1.1 to 4.3 hours. The HMG-CoA reductase inhibitor simvastatin impaired thrombin induced TF expression, and BBR blunted this inhibition. Simvastatin did not affect TNF-α induced TF expression, and BBR enhanced TF under these conditions. Administration of BBR (100 mg/kg/d) increased TF activity and impaired TFPI expression in carotid artery of $ApoE^{-/-}$ mice.

Conclusions. BBR enhances TF via mRNA stabilization at clinically relevant concentrations. Clinical application of BBR, either as an alternative to or in combination with statins, should be considered with caution.

Key Words: Berberine, Tissue Factor, Tissue Factor Pathway Inhibitor, mRNA Stability, Simvastatin

Introduction

Arterial thrombosis is the critical event in acute coronary syndromes, peripheral ischemia, and stroke. Tissue factor (TF) plays an essential role in coagulation by binding factor VII, which activates factor X, finally leading to thrombin generation [1]. TF expression is detected in a variety of cell types within the atherosclerotic vessel wall and is induced by inflammatory mediators such as TNF-α, histamine, or lipopolysaccharide [2,3,4,5]. Elevated levels of TF are indeed present in plaques from patients with unstable angina and enhance plaque thrombogenicity; further, an involvement of TF in drug-eluting stent thrombosis has been discussed as well [6,7,8]. Tissue factor pathway inhibitor (TFPI) acts as the direct endogenous inhibitor of the TF/FVIIa complex [9]. Increasing evidence indicates that modulation of the physiological balance between TF and TFPI has an important impact on thrombus formation [10,11].

Berberine (BBR), an alkaloid isolated from the Chinese herb huanglian (Coptis chinensis), has been extensively used in traditional Chinese medicine. Recently, BBR was identified as a promising lipid-lowering drug, potently upregulating hepatic low-density lipoprotein (LDL) receptor expression. This effect occurred via LDL receptor mRNA stabilization rather than increased promoter activity and required extracellular signal regulated kinase (ERK) activation [12]. Further studies revealed that the BBR induced LDL receptor mRNA stabilization involved novel regulatory proteins located downstream of the ERK pathway and able to interact with sequences in the proximal section of the LDL receptor mRNA 3’ untranslated region (UTR) [13].

Since these observations suggest a therapeutic application of BBR, either as a

monotherapy or in combination with statins, this study adressed the question whether BBR, similar to statins, exerts pleiotropic effects on endothelial TF expression.

Methods

Cell Culture

Human aortic endothelial cells (HAEC; Clonetics, Allschwil, Switzerland) were cultured as described [3,4]. Adhering cells were grown to confluence in 3 cm dishes and rendered quiescent in medium supplemented with 0,5% fetal bovine serum for 24 hours before stimulation with 5 ng/mL TNF-α (R&D Systems, Minneapolis, MN) or 1 U/ml thrombin (Sigma, St. Louis, MO). Cells were treated with BBR (Cayman Chemical, Ann Arbor, MI), simvastatin (Sigma), or both for 60 min prior to stimulation. Cytotoxicity was assessed with a colorimetric assay for detection of lactate dehydrogenase release (Roche, Basel, Switzerland).

Western Blot

Protein expression was determined by Western blot analysis as described [3,4]. Antibodies against human TF and TFPI (both from American Diagnostica, Stamford, CT) were used at 1:2000 dilution. Antibodies against phosphorylated p38 MAP kinase (p38), p44/42 MAP kinase (extracellular signal regulated kinase [ERK]), and c-Jun NH2-terminal kinase (JNK; all from Cell Signaling, Danvers, MA) were used at 1:1000, 1:5000, and 1:1000 dilution, respectively. Antibodies against total p38, ERK, and JNK (all from Cell Signaling) were used at 1:2000, 1:5000, and 1:1000 dilution, respectively. All blots were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression (1:5000 dilution, Chemicon International, Temecula, CA).

TF Surface Activity

TF activity at the surface of HAEC was analyzed using a colorimetric assay

(American Diagnostica) [3,4]. TF/FVIIa complex converted human factor X to factor Xa, which was measured by its ability to metabolize a chromogenic substrate. TF activity was measured against a standard curve performed with lipidated human TF to assure that measurements were taken in the linear range of detection.

Real Time PCR

Total RNA was extracted from HAEC using TRIzol Reagent (Invitrogen, Carlsbad, CA). Conversion of total cellular RNA to cDNA was carried out with Moloney murine leukemia virus reverse transcriptase and random hexamer primers (Amersham Biosciences, Piscataway, NJ) in a final volume of 33 µl using 4 µg of cDNA. Real time PCR was performed in an MX3000P PCR cycler (Stratagene, Amsterdam, The Netherlands) as described [7,8]. All experiments were performed using the SYBR Green JumpStart kit (Sigma). Each reaction (25 µl) contained 2 µl cDNA, 1 pmol of each primer, 0.25 µl of internal reference dye, and 12.5 µl of JumpStart Taq ReadyMix (containing buffer, dNTPs, stabilizers, SYBR Green, Taq polymerase and JumpStart Taq antibody). The following primers were used: for full length human TF (F3): sense 5'-TCCCCA-GAGTTCACACCTTACC–3', antisense 5'–CCTTTCTCCTGGCCCATACAC–3' (bases 843-863 of F3 cDNA; NCBI no. NM 001993); for human ribosomal L28: sense 5'-GCATCTGCAATGGATGGT-3', antisense 5'–CCTTTCTCCTGGCCCATACAC-3'. The amplification program consisted of 1 cycle at 95˚C for 10 min, followed by 35 cycles with a denaturing phase at 95˚C for 30 s, an annealing phase at 60˚C for 1 min, and an elongation phase at 72˚C for 1 min. A melting curve analysis was performed after amplification to verify the accuracy of the amplicon, and PCR products were analyzed on an ethidium bromide stained 1% agarose gel. In each real-time PCR run for TF and L28,

a calibration curve was included that was generated from serial dilutions of purified amplicons.

TF Promoter Activity

The TF promoter (-227 bp to +121 bp) was inserted upstream of the luciferase cDNA and the SV40 PolyA signal into the multiple cloning site of the helper vector VQAd5K-NpA (provided by ViraQuest Inc., North Liberty, IA). In a first step, HindIII and BamHI restriction sites of VQAd5K-NpA were used to insert a 2.7 kb HindIII/BamHI restriction fragment of pGL2-Basic vector (Promega, Madison, WI) containing the luciferase cDNA and the SV40 PolyA signal. In a second step, a 0.3 kb KpnI restriction fragment from a human TF promoter plasmid including the TF minimal promoter [14] kindly provided by Dr. Nigel Mackman (University of North Carolina, Chapell Hill; NC) was ligated into the KpnI site of the resulting construct. The whole insert was sequenced to confirm its orientation and the absence of any nucleotide substitutions. This construct named VQAd5/hTF/Luc was used for production of an adenoviral vector (Ad5/hTF/Luc). For transduction, the vector was added to HAEC at an moi of 60 pfu/cell for 1 hour and then removed. HAEC were kept in growth medium for 24 hours and then serum-starved for 24 hours prior to TNF-α stimulation with or without BBR (30 μmol/L) for 5 hours. Firefly luciferase activity was determined in cell lysates using a luminometer (Berthold Technologies, Bad Wildbad, Germany). Protein concentration of the cell lysates was determined for normalization of luciferase activity.

TF mRNA Stability

2 hours after TNF-α stimulation, transcription was stopped by addition of actinomycin D (Sigma, St. Louis, MO) at a concentration of 10 μg/ml. At the indicated

time points following addition of actinomycin D, cells were washed with PBS and immediately lysed with TRIzol reagent for RNA isolation. RNA was processed for real-time PCR analysis as described above. Values were then plotted on a logarithmic scale as a function of time, and the half-life was calculated using linear equation [15].

In vitro RNA decay assay

A 297-bp PCR fragment from 3'-UTR of the human TF cDNA was subcloned into the BamHI and XhoI restriction sites of pBluescript II KS (+) (Stratagene). This construct was linearized with KpnI and used as a template for generation of a 390-nt riboprobe labeled with 50 µCi [α-^{32}P]UTP (25 Ci/mmol, Perkin Elmer) using T7 RNA polymerase (Promega) and m^{7}G5'pppG (cap). The labeling reaction was performed for 1 h at 30°C followed by removal of the DNA template through incubation with RQ1 RNase-Free DNase for 15 min at 37°C. The riboprobe was then purified on 6% Tris boric acid-EDTA buffer-urea polyacrylamide gel. Cytoplasmic proteins were extracted in lysis buffer (25 mM HEPES, pH 7.6, 5 mM MgCl2, 1.5 mM KCl, 2 mM DTT, 1 mM PMSF, 0.1% NP-40). ^{32}P-labeled riboprobe (10^5 cpm) was added to 20 µg cytoplasmic protein extracts (1 µg protein/µl) in buffer (20 mM HEPES (pH 7.0), 20% glycerol, 100 mM *KCl*, 0.2 mM EDTA, 1 mM DTT, 1mM PMSF), and this mixture incubated at room temperature in RNA-binding buffer (20 µl) containing 20 mM HEPES (pH 7.0), 20% glycerol, 100 mM KCl, 3 mM $MgCl_2$, 2 mM DTT, 0.5 % NP-40, yeast RNA (1 µg), and heparin (1 µg). Aliquots were removed at 0 min, 15 min, 30 min, and 60 min time-points and placed in 100 µl stop solution containing 25 mM Tris (pH 7.6), 400 mM NaCl, 0.1 % SDS, and yeast RNA (10 µg). Samples were extracted with phenol-cloroform, precipitated with ethanol, and separated by gel-

electrophoresis on 5% polyacrylamide gel containing 7M urea. Bands were visualized by autoradiography using a phosphoimager.

In vivo study

For analysis of TF and TFPI in vivo, 10 week old male ApoE$^{-/-}$ mice (C57BL6, Jackson Laboratories, Bar Harbor, ME) weighing an average of 28g were fed a normal chow diet (KLIBA NAFAG, Kaiseraugst, Switzerland) and treated with BBR at a dose of 100 mg/kg/d orally for 10 days*. Control ApoE$^{-/-}$ mice matched for age, sex, and weight received an equal volume of vehicle (0.9% saline).* Mice were then euthanized and the left common carotid artery harvested for analysis of TF activity and TFPI expression. Analysis of TF activity in the arterial homogenates was performed using a colorimetric assay (American Diagnostica). TFPI expression was assessed by ELISA (American Diagnostica).

Statistics

Data are presented as mean±SEM. Statistical analysis was performed by ANOVA or 2-tailed unpaired Student t-test as appropriate. A value of $p<0.05$ was considered significant. All results are representative of at least 4 independent experiments.

Results

Berberine enhances TF protein expression and surface activity

HAEC were stimulated with TNF-α (5 ng/ml) or thrombin (1 U/ml) for 5 hours. 1 hour pretreatment with BBR (1-30 μmol/l) enhanced TNF-α induced TF expression in a concentration-dependent manner with an EC50 value of 2 μmol/l (n=5; $p<0.01$; Figure 1A); the maximal effect occurred at 30 μmol/l and resulted in a 3.4-fold higher expression as compared to TNF-α alone. Similarly, BBR enhanced thrombin induced TF expression by 3.3-fold as compared to thrombin alone (n=5; $p<0.05$; Figure 1B). BBR did not alter TF expression under basal conditions (n=10; p=NS; Figure 1A and B). The effect of BBR on TF expression was paralleled by an enhanced TF surface activity, which was 1.6 times higher than that by TNF-α alone (n=4; $p<0.0005$; Figure 1C). BBR did not alter TF surface activity under basal conditions (n=4; p=NS; Figure 1C).

BBR (1-30 μmol/l) inhibited TFPI expression of TNF-α stimulated HAEC by 45±15% as compared to TNF-α alone with an EC50 value of 5.7 μmol/l (n=4; $p<0.05$; Figure 1D). Similarly, BBR impaired basal TFPI expression by 52±12% (n=4; $p<0.005$; Figure 1D).

LDH release was not affected by any BBR concentration used (n=4; p=NS; data not shown) indicating that BBR did not exert cytotoxic effects.

Berberine enhances TF mRNA level

Real-time PCR revealed that TNF-α induced TF mRNA expression within 2 hours of stimulation (n=5; $p<0.0001$; Figure 2A). BBR (30 μmol/l) enhanced TNF-α induced TF mRNA expression by 1.6-fold as compared to TNF-α alone (n=5; $p<0.05$;

Figure 2A).

Berberine inhibits TF promoter activity

To assess whether the effect on TF mRNA was mediated by enhanced transcription, the impact of BBR on TF promoter activity was analyzed after transfection of HAEC with a plasmid expressing firefly luciferase under control of the human TF promoter (-221bp to +121bp). TNF-α enhanced TF promoter activity by 2.9-fold as compared to control conditions (n=4; $p<0.0001$; Figure 2B). Treatment with BBR (30 µmol/l) impaired promoter activation (n=4; $p<0.001$; Figure 2B). BBR did not affect basal promoter activity (n=4; p=NS; Figure 2B).

Berberine inhibits JNK phosphorylation

To determine whether inhibition of TF promoter activity was paralleled by inhibition of MAP kinases, phosphorylation of these mediators was assessed at different time points after TNF-α stimulation. TNF-α (5 ng/mL) transiently activated JNK, ERK, and p38; maximal activation was observed after 15 minutes and returned to basal levels within 60 minutes (Figure 3A). Treatment with BBR inhibited phosphorylation of JNK by 48±12% as compared to TNF-α alone (n=4; $p<0.05$; Figure 3B), while phosphorylation of ERK and p38 was not significantly reduced (n=4; p=NS; Figure 3B). Total expression of JNK, ERK, and p38 remained unaffected at all time points examined (Figure 3A).

Berberine stabilizes TF mRNA

TF mRNA was quantified by real-time PCR at different time points after addition of the inhibitor of transcription, actinomycin D. BBR (30 µmol/L) delayed TF mRNA degradation, increasing the half-life of TF mRNA from 64 min under control

conditions to 260 min in the presence of BBR (n=5; Figure 4A). To study the role of the 3'-untranslated region (3'-UTR), which includes five AU-rich elements known to mediate TF mRNA stability, an in vitro RNA decay assay was performed. A ^{32}P-radiolabeled 390 nt transcript containing the last 297 nt of the TF 3'-UTR was incubated with cytoplasmatic extracts generated from endothelial cells either untreated or treated with TNF-α, berberine, or TNF-α+berberine. Analysis of transcript decay by densitometry showed no significant difference in transcript degradation rate between the different groups (n=4; p=NS; Figure 4B).

Berberine abrogates the inhibitory action of statins on TF expression

Statins exert pleiotropic anti-thrombotic effects through inhibition of TF expression in different cell types including endothelial cells [16,17,18]. Since statins and BBR exhibit additive lipid-lowering effects, the impact of such a combination on endothelial TF protein expression was examined. Simvastatin (1 μmol/l) inhibited thrombin induced TF expression by 40±14% as compared to thrombin alone (n=5; $p<0.05$; Figure 5A), while such an effect was not observed in TNF-α stimulated endothelial cells (n=4; p=NS for TNF-α + simvastatin vs TNF-α alone; Figure 5B). BBR antagonized the inhibitory effect of simvastatin on thrombin induced TF expression (n=5; $p<0.05$ for simvastatin + BBR vs simvastatin alone; p=NS for simvastatin + BBR vs BBR alone; Figure 5A). In both thrombin and TNF-α stimulated HAEC, treatment with simvastatin had no effect on the enhanced TF expression occurring in the presence of BBR (n=4; p=NS; Figure 5B).

BBR enhances TF and inhibits TFPI in vivo

The effect of berberine on expression of TF and TFPI was assessed in ApoE$^{-/-}$ mice. Animals were treated for 10 days with berberine (100 mg/kg/d). The left

common carotid artery was harvested for analysis of TF activity and TFPI expression. TF activity was enhanced by nearly 2-fold in the berberine treated group ($n=7$; $p<0.05$; Figure 6A). This increase in TF activity was paralleled by a 43.5% decrease in TFPI expression as compared to control ($n=7$; $p<0.05$; Figure 6B).

Discussion

This study demonstrates that BBR, at clinically relevant concentrations stimulating hepatic LDL receptor expression [12], enhances TNF-α and thrombin induced endothelial TF expression via stabilization of TF mRNA. Moreover, BBR inhibits endothelial TFPI expression, and abrogates the inhibitory effects of statins on endothelial TF. Similar effects on TF and TFPI are observed in $ApoE^{-/-}$ mice treated with BBR. These observations suggest that application of BBR as a lipid-lowering medication, either alone or in combination with a statin, may favor the development of thrombosis, in particular in the inflammatory environment of atherosclerotic lesions [19].

As endothelial cells are situated at the luminal surface of vessels and thereby exposed to circulating FVII, modulation of TF expression at the endothelial surface can have major consequences for thrombus formation. BBR enhanced TNF-α and thrombin induced TF expression, suggesting that it promotes thrombus formation under both inflammatory and prothrombotic conditions. Since TF is inhibited by TFPI, the balance of these factors is essential for vascular homeostasis [9,10,20]. In addition to enhancing TF, BBR impaired basal as well as TNF-α induced endothelial TFPI expression, which represents an additional prothrombotic potential. These effects of BBR on TF and TFPI may be particularly relevant in acute coronary syndromes, which are usually triggered by vascular inflammation leading to thrombus formation.

The BBR concentrations applied in vitro (1 to 30 μmol/l) are comparable to those used in another study [12], where concentrations ranged from 0.5 to 15 μg/ml corresponding to 1.3 to 40 μmol/l. The pharmacokinetics of berberine in humans are

barely understood and still need to be characterized. However, a study performed in patients with congestive heart failure reported that oral administration of 1.2 g BBR per day led to plasma concentrations of 190±0.08 ng/ml (0.5±0.03 μmol/l) [21]. For the in vivo animal studies performed by others as well as for our own in vivo experiments, BBR was administrated orally at a dose of 50 and 100 mg/kg/d corresponding to an oral intake of 3 and 6 g BBR per day, respectively, for an adult weighing 60 kg [12]; hence, BBR intake was higher than in the study performed in humans, suggesting that BBR plasma concentrations may well reach values of 2 μmol/l under these conditions. The berberine mediated effects on endothelial TF were statistically significant at concentrations as low as 1 μmol/l, and consistent with a biologically relevant concentration range, the in vivo effects of BBR on TF and TFPI were similar to those observed in human cells.

The MAP kinases JNK, ERK, and p38 are critically involved in mediating transcriptional regulation of TF expression in response to both TNF-α and thrombin [1]. Although BBR enhanced TF mRNA levels, it inhibited activation of JNK without affecting that of ERK and p38, and, in line with this observation, impaired TF promoter activation. These data indicate that BBR upregulates TF mRNA by a post-transcriptional mechanism able to counteract the impaired TF promoter activation. Analysis of TF mRNA decay rates indeed confirmed that BBR prolonged the half life of TF mRNA from 64 to 260 minutes, highlighting the crucial role of mRNA stability in the regulation of TF expression. A similar effect on the half life of TF mRNA was observed with dexamethasone in human monocytes [5,22]. The importance of mRNA stability in mediating TF expression, however, varies depending on the stimulus involved; indeed, LPS induces TF mainly by enhancing mRNA stability rather than increasing the rate of transcription, while the opposite effect accounts for the action of PMA [23]. Hence, it is conceivable that the mechanism of action of BBR on

endothelial TF expression differs depending on the stimulus activating the endothelium.

In mammalian cells, AU sequences regrouped to AUUUA pentamers in so-called AU-rich elements (ARE) located in the 3' untranslated region of mRNA are known to modulate mRNA decay [12,24,25]. ARE have been described in the 3'-UTR of human TF mRNA and are involved in TF mRNA turnover [22,26]. Analysis of the decay rates of a 390 nt transcript containing the last 297 nt of the TF 3'-UTR suggested that the stabilizing effect of berberine on TF mRNA is not mediated by elements within the last 297 nt of the TF 3'-UTR since there was no difference in degradation rates of the transcript in the presence of cytoplasmatic extracts from cells treated with or without BBR. 3'-UTR ARE and their binding proteins are not the only regulators of mRNA stability; other mechanisms such as inhibition of translational elongation or the presence of stabilizing sequences located in the 5'-UTR can modulate mRNA decay as well [23,24]. So far, however, it remains unknown whether the latter mechanisms are involved in regulating TF mRNA stability.

Statins are among the most-widely prescribed drugs, and the indications for their prescription have been extended over the years. Besides their well established lipid-lowering properties, statins exert cardio-protective pleiotropic effects [27,28]. Simvastatin indeed inhibited thrombin induced endothelial TF expression; however, TNF-α induced TF expression was not affected, indicating that such pleiotropic effects are modulated by the mediators involved in endothelial activation. Since another statin, cerivastatin, prevented TNF-α induced TF expression in human endothelial cells [29], this class of drugs may be heterogenous with regard to modulation of TF expression; alternatively, it should be considered that this experiment was performed in human umbilical vein endothelial cells and that

experimental conditions might vary. BBR blunted the inhibitory effect of simvastatin on thrombin induced TF expression; moreover, it enhanced TF expression in cells treated with either TNF-α alone or TNF-α together with simvastatin. Both effects can be explained by the posttranscriptional mechanisms through which BBR enhances TF expression.

In summary, this study reveals that the natural lipid-lowering drug, BBR, enhances TF expression and in parallel decreases TFPI expression both in human endothelial cells and in arteries of $ApoE^{-/-}$ mice. Hence, BBR may favor the development of thrombus formation, particularly in patients with atherosclerotic lesions and those with acute coronary syndromes, since prothrombotic and inflammatory environments are often encountered under these conditions. Therefore, clinical application of BBR should be considered with caution, at least until large-scale clinical trials have proven its safety.

Acknowledgements

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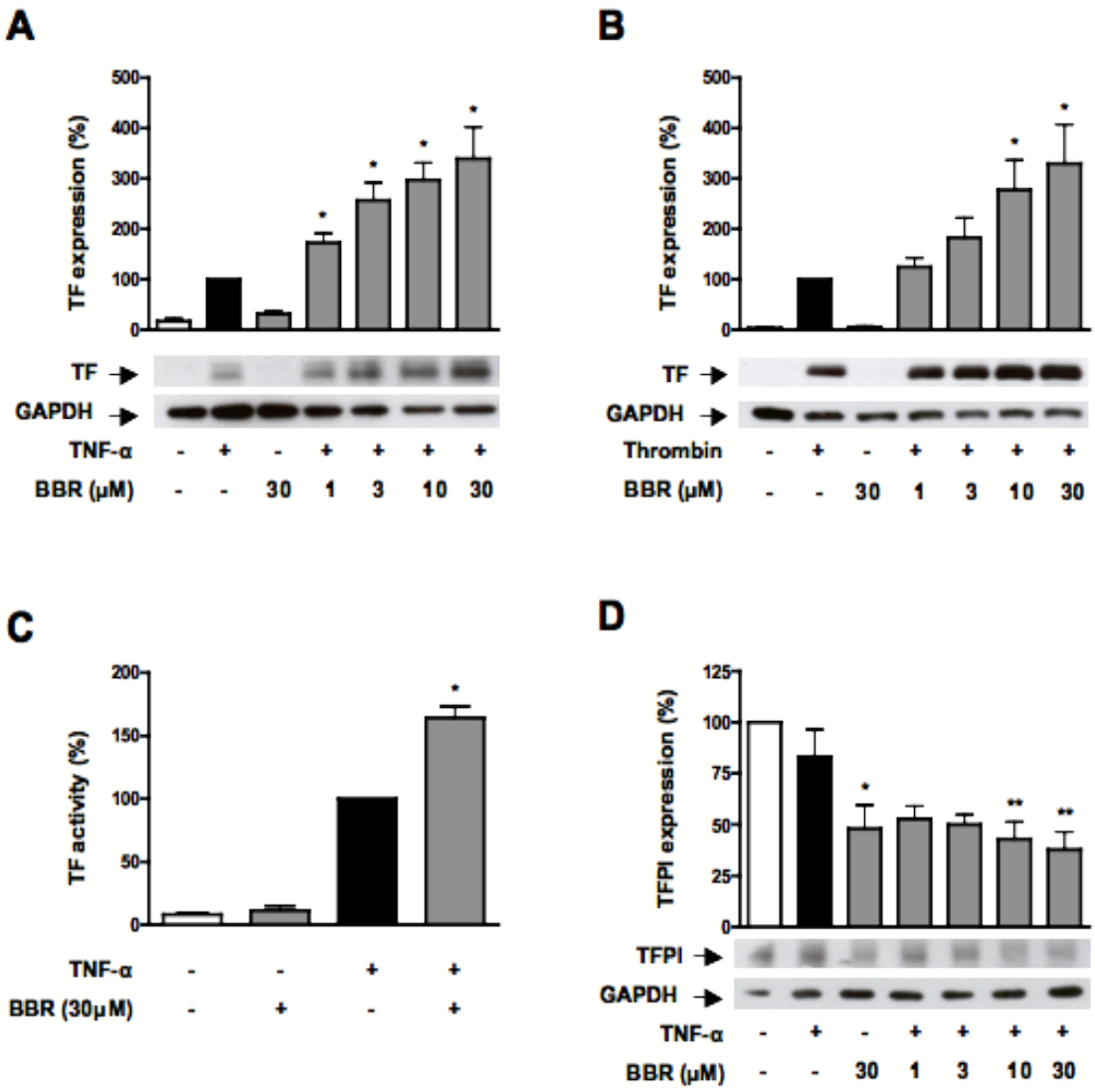
A
TF expression (%)
500
400
300
200
100
0
TF
GAPDH
TNF-α - + - + + + +
BBR (μM) - - 30 1 3 10 30
B
TF expression (%)
500
400
300
200
100
0
TF
GAPDH
Thrombin - + - + + + +
BBR (μM) - - 30 1 3 10 30
C
TF activity (%)
200
150
100
50
0
TNF-α - - + +
BBR (30μM) - + - +
D
TFPI expression (%)
125
100
75
50
25
0
TFPI
GAPDH
TNF-α - + - + + + +
BBR (μM) - - 30 1 3 10 30

Figure 1

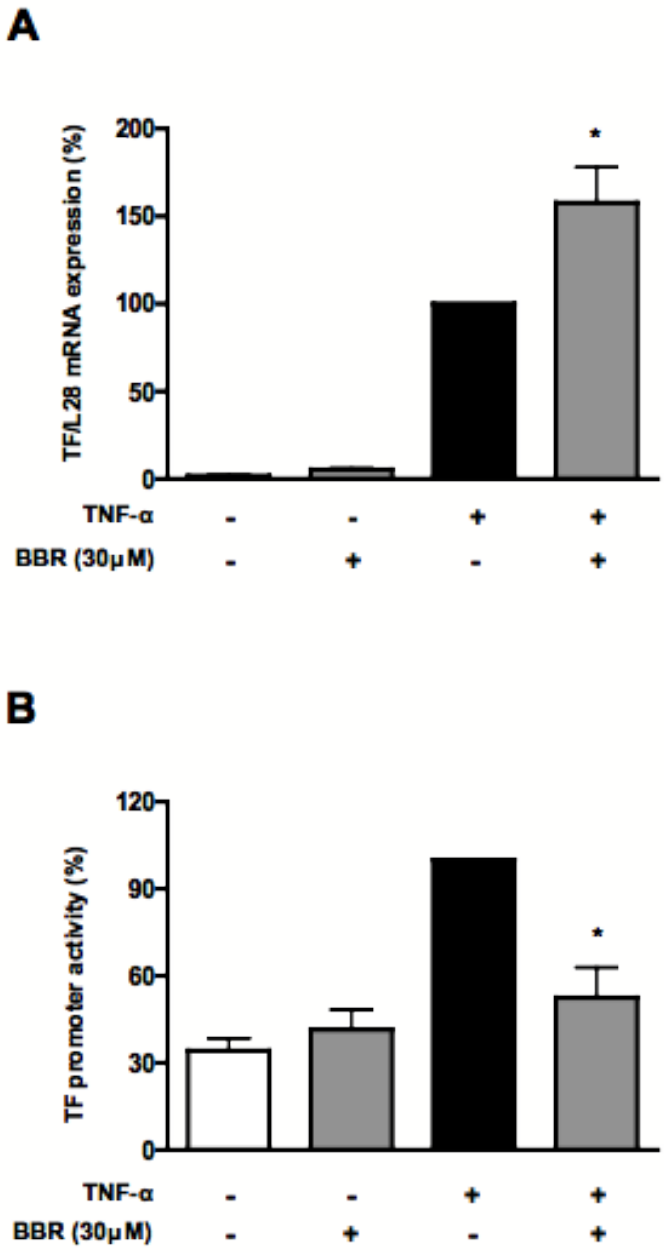
A
TF/L28 mRNA expression (%)
200
150
100
50
0
*
TNF-α - - + +
BBR (30μM) - + - +
B
TF promoter activity (%)
120
90
60
30
0
*
TNF-α - - + +
BBR (30μM) - + - +

Figure 2

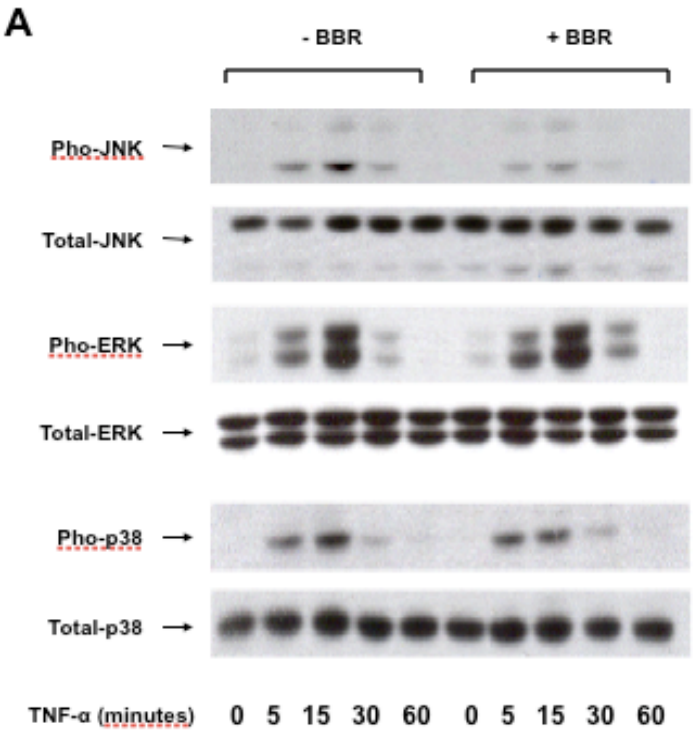
A
- BBR
+ BBR
Pho-JNK
Total-JNK
Pho-ERK
Total-ERK
Pho-p38
Total-p38
TNF-α (minutes) 0 5 15 30 60 0 5 15 30 60
B
JNK
ERK
p38
TNF-α (15 min) + + +
BBR (30μM) + + +
MAP kinase inhibition (%)
0
20
40
60
80
*

Figure 3

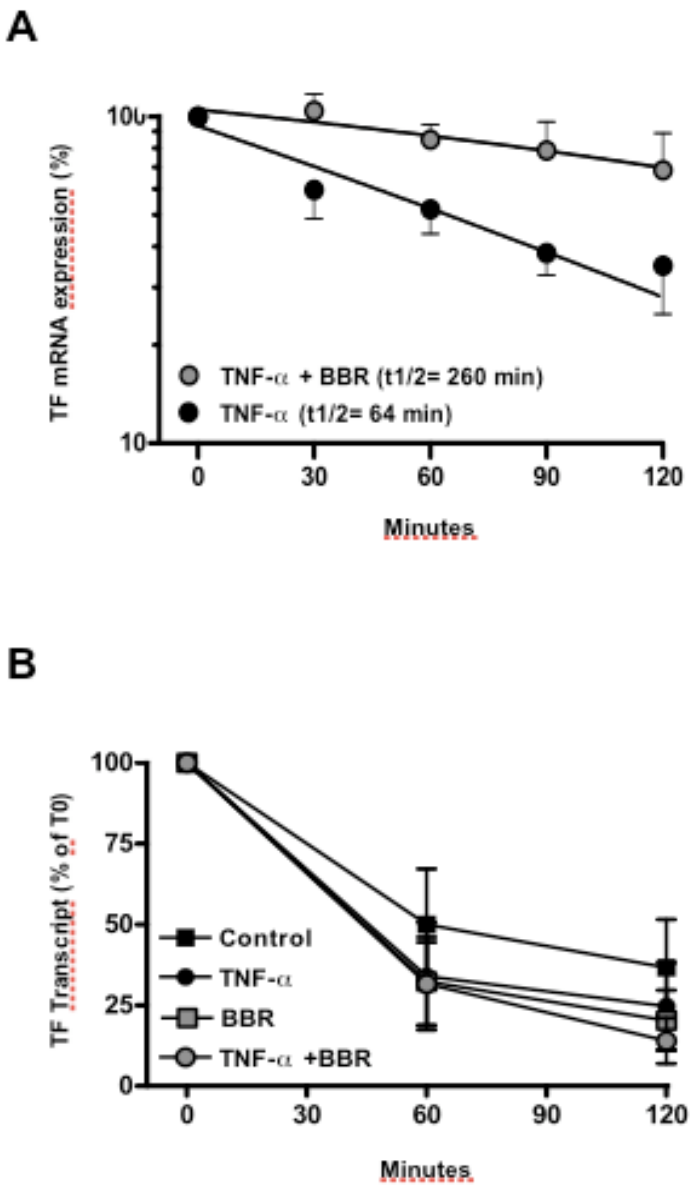
A
100
10
TF mRNA expression (%)
TNF-α + BBR (t1/2= 260 min)
TNF-α (t1/2= 64 min)
0
30
60
90
120
Minutes
B
100
75
50
25
0
TF Transcript (% of T0)
Control
TNF-α
BBR
TNF-α +BBR
0
30
60
90
120
Minutes

Figure 4

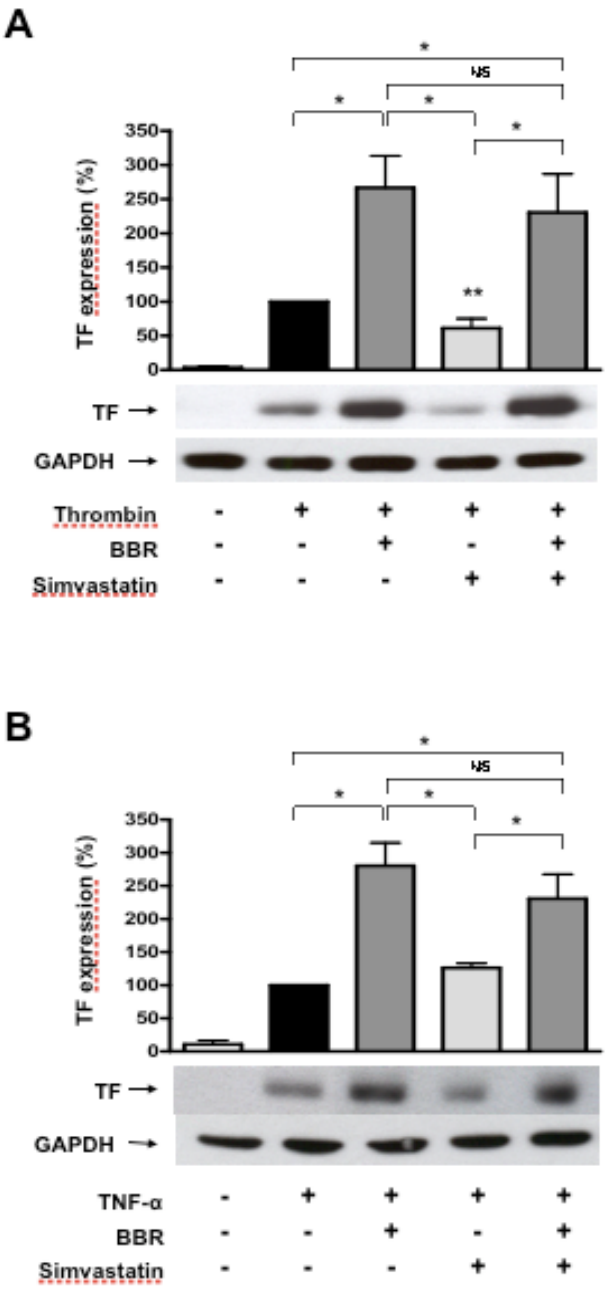
A
TF expression (%)
350
300
250
200
150
100
50
0
*
NS
*
*
*
**
TF
GAPDH
Thrombin - + + + +
BBR - - + - +
Simvastatin - - - + +
B
TF expression (%)
350
300
250
200
150
100
50
0
*
NS
*
*
*
TF
GAPDH
TNF-α - + + + +
BBR - - + - +
Simvastatin - - - + +

Figure 5

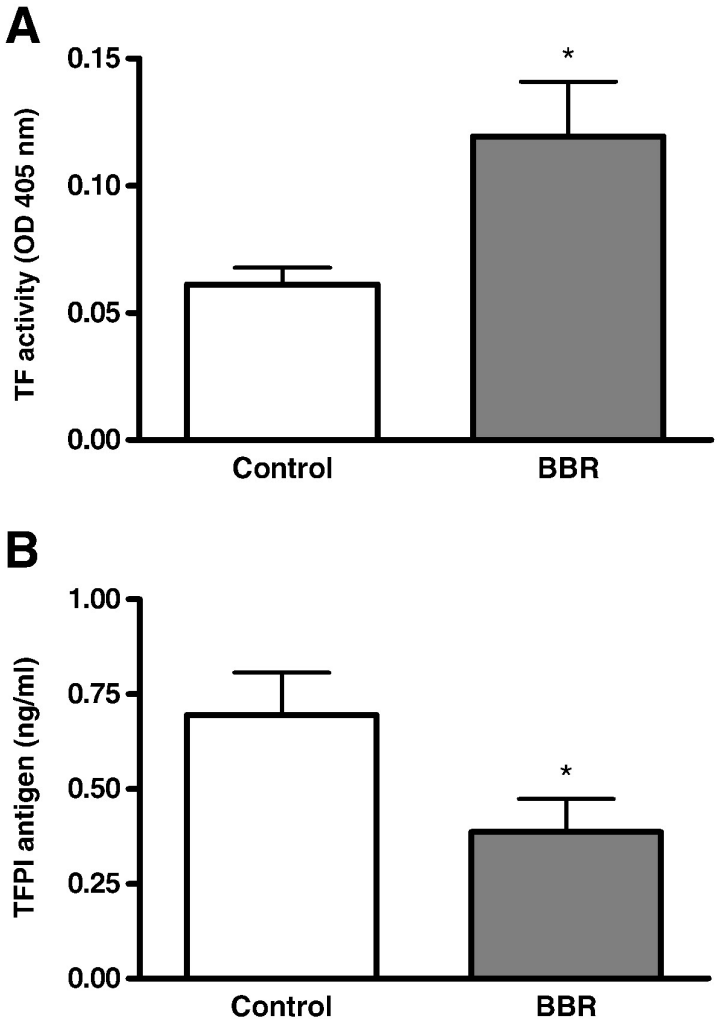
A
0.15
0.10
0.05
0.00
TF activity (OD 405 nm)
*
Control
BBR
B
1.00
0.75
0.50
0.25
0.00
TFPI antigen (ng/ml)
*
Control
BBR

Figure 6

Figure Legends

Figure 1: BBR enhances TF protein expression and surface activity, and inhibits TFPI expression

Western blot analysis demonstrates that BBR enhances TNF-α **(A)** and thrombin **(B)** induced TF protein expression. *p<0.01 vs TNF-α alone, *p<0.05 vs thrombin alone. **C.** BBR enhances TNF-α induced TF surface activity. *p<0.001 vs TNF-α alone. **D.** BBR inhibits TFPI protein expression. *p<0.005 vs control; **p<0.05 vs. TNF-α alone. All blots are normalized to GAPDH expression.

Figure 2: BBR enhances TF mRNA expression, but inhibits TF promoter activation.

A. Real-time PCR demonstrates that BBR enhances TNF-α induced TF mRNA expression. Values are normalized to L28 expression. *p<0.005 vs TNF-α alone. **B.** BBR inhibits TNF-α induced TF promoter activation. Values are normalized to total protein concentration. *p<0.001 vs TNF-α alone.

Figure 3: BBR inhibits JNK phosphorylation.

A. TNF-α leads to transient phosphorylation (Pho) of JNK, ERK, and p38. BBR inhibits activation of JNK, but not ERK and p38. Total (tot) expression of JNK, ERK, and p38 remains unchanged. **B.** BBR inhibits phosphorylation of JNK after 15 minutes of TNF-α stimulation. *p<0.05 vs TNF-α alone.

Figure 4: BBR stabilizes TF mRNA

A. The decay of TF mRNA after addition of actinomycin D is monitored by real

time PCR and normalized to L28 mRNA. Normalized TF mRNA levels are plotted in a semi-logarithmic scale as percentage of mRNA remaining versus time. The half-lives of TF mRNA are indicated. **B.** In vitro RNA decay assay reveals that the 3'UTR is not involved in the effect of BBR on TF mRNA. p=NS vs control.

Figure 5: BBR abrogates TF inhibition by simvastatin

A. Simvastatin inhibits thrombin induced TF expression as determined by western blot.**p<0.05 vs thrombin alone. BBR abrogates this effect. *p<0.05 vs thrombin alone, *p<0.05 vs thrombin + simvastatin. **B.** Simvastatin does not affect TNF-α induced TF expression. p=NS vs TNF-α alone. BBR enhances TF expression in the presence of simvastatin. *p<0.05 vs TNF-α alone, *p<0.05 vs TNF-α + simvastatin.

Figure 6: BBR enhances TF and inhibits TFPI in vivo

A. BBR enhances TF activity in ApoE-/- mice. *p<0.05 vs control. **B**. BBR inhibits TFPI expression in ApoE-/- mice. *p<0.05 vs control.

VI. Manuscript in preparation

PI3K/p110α Inhibition Differentially Regulates Vascular Smooth Muscle and Endothelial Cell Proliferation and Suppresses Prothrombotic Endothelial Cell Activation: Novel Implications for Drug-Eluting Stent Design

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Abstract

Objectives. To evaluate the impact of p110α inhibition on vascular smooth muscle (VSMC) and endothelial cell (EC) activation.

Background. Impaired reendothelialization and stent thrombosis remain safety concerns associated with the use of drug-eluting stents DES, despite a reduction in restenosis rates. Phosphoinositide 3-kinase p110α (PI3K/p110α) controls crucial cellular processes including proliferation and chemotaxis, thereby representing an emerging drug target. However, its effect on vascular smooth muscle (VSMC) and endothelial cell (EC) activation remains unknown.

Methods. PI3K/p110α was inhibited by treatment with the small molecule inhibitor PIK 75 or, alternatively, a specific siRNA. Proliferation and migration of VSMC and EC were assessed by cell number and Boyden chamber, respectively. Endothelial senescence and dysfunction were evaluated by β-galactosidase assay, Western blots for expression of eNOS, TF, and PAI-1, and organ chambers for isometric tension recording.

Results. Inhibition of PI3K/p110α with PIK 75 or a specific siRNA selectively impaired proliferation and migration of VSMC while sparing EC completely. Treatment with PIK75 did not induce endothelial senescence nor inhibit eNOS expression or endothelium-dependent vascular relaxation. However, PIK 75 inhibited both basal and TNF-α induced expression of TF and PAI-1. In contrast to PIK 75, both rapamycin and paclitaxel inhibited endothelial proliferation and migration; moreover they induced expression of TF and PAI-1.

Conclusions. Inhibition of PI3K/p110α impairs proliferation and migration of VSMC, but not EC. In addition to its potent antiproliferative and antimigratory effects on

VSMC, targeting p110α inhibits the expression of prothrombotic mediators on EC. Hence, PI3K/p110α inhibition may offer new options in DES design.

Introduction

Revascularization by percutaneous transluminal coronary angioplasty (PTCA) and subsequent deployment of balloon-expendable stents has become the treatment of choice in symptomatic coronary artery disease. Because of procedure-related vascular injury and remodeling associated with high restenosis rates, stents designed to release anti-proliferative pharmacological agents have been developed. The introduction of rapamycin- and paclitaxel-eluting stents has resulted in a significant reduction of restenosis and target-vessel revascularization rates (TLR) when compared with bare-metal stents (BMS) (1-4). Despite this success, drug-eluting stents (DES) have failed to improve mortality when compared to BMS and recent trials have raised concerns over increased stent thrombosis rates associated with the use of DES (5-7). Indeed, impaired reendothelialization as well as prothrombotic effects of the eluted drug seem to play important roles in the pathogenesis of stent thrombosis observed with DES (8). Therefore the ideal DES should exert selective anti-proliferative effects on vascular smooth muscle cells, while being inert toward endothelial cell proliferation (9).

By catalyzing the synthesis of phosphatidynol second messengers (PIP, PIP_2, and PIP_3), the phosphatidylinositol-3 kinase (PI3K) signaling pathway controls fundamental cellular processes such as cell survival, proliferation and differentiation (10). The class I PI3Ks consist of a dimer of a regulatory (p85α) and a catalytic subunit (p110α, p110β, and p110δ). Class I PI3ks are activated by tyrosine kinases or G- protein coupled receptors to catalyze the generation of PIP_3, which modulates downstream targets such as the Rho GTPases or the Akt/PDK1 pathway (10). The implication of the PI3Ks in pathophysiological processes such as cancer

development, inflammation, or thrombosis has made these enzymes an attractive therapeutic target and recent efforts have been taken to synthesize cell-permeable small molecule PI3K inhibitors such as the p110α inhibitor PIK 75 (11,12). However, because of their ubiquitous distribution, the understanding of how the several PI3K isoforms control normal physiological processes remains an important issue in the development of PI3K-targeting drugs.

In the vasculature, p110α is the predominant isoform expressed in endothelial (EC) and vascular smooth muscle cells (VSMC). This study was designed to address the role of p110α in EC and VSMC proliferation and migration, which are critically involved in vascular remodelling following coronary intervention.

Methods

Cell culture

Human aortic endothelial (HAEC), aortic smooth muscle (AoSMC) and human umbilical vein endothelial cells (HUVEC; all from Lonza, Basel, Switzerland) were cultured as described (13,14). Saphenous vein smooth muscle cells (SVSMC) were isolated from human saphenous veins, characterized, and cultured as described (15). Briefly, AoSMC and SVSMC were grown on flasks (BD Biosciences, Bedford, MA, USA) in Dulbecco´s modified Eagle´s medium (DMEM) (Invitrogen, Basel, Switzerland) plus 10% fetal calf serum (FCS) using passages 5 to 9. HAEC and HUVEC were grown on fibronectin-coated flasks in endothelial cell growth medium-2 (EGM-2) (Lonza) containing 10% FCS.

Proliferation assay

For cell counts AoSMC and SVSMC were seeded at a density of 15 000 cells per 35 mm dish and cultured in DMEM plus 10% FCS for 1 day. After 48 hours of serum withdrawal (DMEM plus 0.2% bovine serum albumin) the cells were incubated with either the small molecule p110α-inhibitor PIK 75 (Axon Biochemicals; Groningen, The Netherlands), paclitaxel, rapamycin, or the vehicle (0.1 % dimethylsulfoxide) (all from Sigma Aldrich, St. Louis, MO, USA) in the presence of platelet derived growth factor-BB (PDGF-BB, 10 ng/ml) and the cells trypsinized and counted 72 hours later.

HAEC and HUVEC were seeded at a density of 20 000 cells per 35 mm dish and grown in EGM-2 plus 10% FCS for 24 hours. After rendering the cells quiescent

in endothelial basal medium-2 (EBM-2) plus 0.1% FCS for 24 hours, the drug and EGM-2 plus 10% FCS were added and the cell number was assessed 72 hours later.

Cell viability was assessed using a colorimetric assay for detection of lactate dehydrogenase release (Roche, Basel, Switzerland).

Migration assay

To assess migration in a 48-well modified Boyden chamber (Neuroprobe, Gaithersburg, MD, USA), AoSMC and SVSMC were seeded at a density of 70-80%, and kept in DMEM (containing 1g/l glucose) plus 10% FCS for 36-48h prior to the assay. Cells were trypsinized, washed with PBS and resuspended in control medium (DMEM plus 0.1% BSA) at a concentration of 500000 cells/ml. For drug pretreatment, cell suspensions were incubated with the corresponding drug for 1h at 37°C. 50µl of cell suspension was loaded into the upper chamber which was separated by a collagen-coated polycarbonate membrane (Neuro Probe, Inc. PFB8; 8µm pores) from the lower chamber containing DMEM (supplemented with 0.1% BSA) plus the chemoattractant PDGF-BB (10 ng/ml). DMEM with PDGF-BB 10 ng/mL without the drug served as positive control, whereas DMEM alone was used as a negative control. Cells were allowed to migrate for 5 hours. Non-migrated cells were removed from the upper surface of the membrane with a cellscraper, and migrated cells on the bottom surface were fixed in ice-cold methanol for 10minutes followed by staining with Diff-Quik (Medion Diagnostics, Dudingen, Switzerland).

For migration assays HAEC und HUVEC were processed the same way as AoSMC and SVSMC; except that they were seeded and kept in EGM-2 plus 10% FCS and resuspended in EBM-2 plus 0.2% FCS as control medium. For all EC's a 5µm pore size collagen-coated polycarbonate membrane was used. EGM-2 plus 10% FCS alone represented the positive control, whereas EBM-2 supplemented only

with 0.1% BSA was used as negative control. Migration for both vascular and endothelial cells was assessed after 5 hours by counting cells in 4 random fields at 200x magnification using an Olympus BX51 microscope (Olympus Europa GmbH; Hamburg, Germany).

siRNA knockdown experiments

HAEC or AoSMC were plated on 6-well dishes 24 hours before transfection at a density of 20 000 and 15 000 cells respectively per well. Four siRNA oligonucleotide sequences (Microsynth, Balgach, Switzerland) reported to target the human p110α catalytic subunit, were used at a concentration of 25 nM and in combination: 1015931 (sense sequence: 5'-GCUAUCAUCUGAACAAUUAUU-3'), 1015933 (sense sequence: 5'-GGAUAGAGGCCAAAUAAUAUU-3'), 1015935 (sense sequence: 5'-GGACAACUGUUUCAUAUAGUU-3'), and 1015937 (sense sequence: 5'-GCCAGUACCUCAUGGAUUAUU-3'). Scrambled siRNA (Microsynth; 1050124; 5'-GAUCAUACGUGCGAUCAGATT-3') was used as control. Transfection was performed using the lipofectamine RNAi Max system (Sigma Aldrich). After transfection, HAEC and AoSMC were transferred to their corresponding growth medium and trypsinized for counting after 72 hours. Knockdown of p110α was confirmed at the protein level by Western blot analysis. Immunoblotting was performed using a human anti-p110α antibody at a dilution of 1:5000 (Cell Signaling Technology, Danvers, MA, USA).

Senescence associated β-galactosidase (SA-β-gal) assay

HAEC were plated on fibronectin coated 6-well dishes and grown for 24 hours to 80% confluence in EGM-2 plus 10% FCS. After replacing the medium (EGM-2 plus 10% FCS), cells were treated with vehicle (0.1% dimethylsulfoxide), PIK 75 (10

nM), rapamycin (10 nM), or paclitaxel (10 nM) for another 24 hours. HAEC were washed 3 times with phosphate buffered saline (PBS) and fixed with 3% paraformaldehyde for 3 minutes. HAEC were incubated with a SA-β-gal stain solution for 14 hours and the proportion of SA-β-gal positive cells determined as previously described.

Analysis of endothelial nitric oxide synthase (eNOS), tissue factor (TF), and plasminogen activator inhibitor-type I (PAI-1) expression

HAEC were plated on 6-well dishes as previously described (13). For analysis of eNOS expression, HAEC were serum starved with EBM-2 plus 0.5% FCS for 24 hours in the presence of PIK 75 (10 nM), rapamycin (10 nM), paclitaxel (10 nM), or the vehicle. For TF and PAI-1 expression analysis, subconfluent HAEC were rendered quiescent for 24 hours and treated with PIK 75 (10 nM), rapamycin (10 nM), paclitaxel (10 nM), or the vehicle for 1 hour prior to stimulation with TNF-α (5 ng/ml; R&D Systems, Minneapolis, MN, USA) for 5 hours. Protein expression was determined by Western blot analysis as described. Cells were lysed in 50 mmol/L Tris buffer, and 35 μg samples were loaded and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred to a polyvinylidene difluoride membrane by semidry transfer. Antibody to eNOS (BD Biosciences), TF (American Diagnostica), and PAI-1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) were used at 1:2000, 1:2000 and 1:5000 dilution, respectively. All blots were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression (1:5000 dilution, Chemicon International, Temecula, CA, USA). The quantification of protein was performed by densitometric analysis using Scion Image Software (Scion Corp., Frederick, MD, USA)

Statistical analysis

Data are presented as mean ± SEM. Statistical analysis was performed by ANOVA. A value of $p<0.05$ was considered significant. All results are representative of at least 4 independent experiments.

Results

Pharmacological inhibition of p110α with PIK 75 inhibits vascular smooth muscle cell but not endothelial cell proliferation.

Incubation of AoSMC and SVSMC with PIK 75 (0.1-10 nM) inhibited PDGF-BB induced proliferation of AoSMC and SVSMC after 72 hours in a concentration dependent manner (n=4; $p<0.05$; figure 1A). In contrast, PIK 75 did not exert any anti-proliferative effect on HAEC and HUVEC (n=4; p=NS; figure 1B). No cytotoxic effect of PIK 75 was observed at any of the concentrations used (n=4; p=NS; data not shown).

PIK 75 inhibits vascular smooth muscle cell but not endothelial cell chemotaxis.

In AoSMC and SVSMC treatment with PIK 75 (10 nM) significantly reduced PDGF-BB induced cell migration (n=5; $p<0.01$; figure 2A). In HAEC and HUVEC, PIK 75 did not affect serum-inuduced chemotaxis (n=5; p=NS; figure 2B).

Silencing of p110α inhibits proliferation of AoSMC but not HAEC.

Incubation of AoSMC and HAEC with siRNA targeting p110α markedly decreased p110α protein expression in both cell types (n=4; $p<0.01$; figure 3A). This decrease in p110α was paralleled by a decreased proliferation of AoSMC (n=4; $p<0.01$ vs. control; figure 3B) but not HAEC (n=4; p=NS vs. control; figure 3B).

PIK 75 exerts similar anti-proliferative and anti-migratory effects in AoSMC as rapamycin and paclitaxel, but not in HAEC.

Treatment with PIK 75 (10 nM), rapamycin (10 nM), or paclitaxel (10 nM) resulted in a comparable inhibition of PDGF-BB (10 ng/ml) induced proliferation and migration (n=4; $p<0.005$; figure 4A) in AoSMC. In HAEC, however, only rapamycin and paclitaxel exerted anti-proliferative and anti-migratory effects while PIK 75 remained inert toward endothelial cell growth and chemotaxis (n=4; $p<0.005$; figure 4B).

PIK 75 has no effect on endothelial senescence and eNOS expression.

Incubation of HAEC with rapamycin (10 nM) and paclitaxel (10 nM) for 24 hours induced a senescent phenotype characterized by an enlarged and elongated cellular morphology. These morphological changes were not observed with PIK 75 (10 nM) (n=4; figure 5A). SA-β-gal assay confirmed enhanced senescence in HAEC treated with rapamycin or paclitaxel in contrast to PIK 75-treated cells (n=4; $p<0.05$; figure 5B). Since eNOS is critically involved in both endothelial senescence and function, we assessed expression of total eNOS in HAEC by Western blot. Rapamycin and paclitaxel, but not PIK 75, decreased total eNOS expression in HAEC after 24h (n=4; $p<0.005$; figure 5C).

PIK 75 inhibits endothelial TF and PAI-1 expression.

Treatment of HAEC with either rapamycin (10 nM) or paclitaxel (10 nM) for 5 hours induced basal TF expression (+47% and +48% respectively; n=4; $p<0.05$ vs. control; figure 6A) and enhanced TNF-α induced TF expression by 70% and 48% respectively (n=4; $p<0.05$ vs. TNF-α alone; figure 6B). In a similar manner, rapamycin and paclitaxel enhanced basal PAI-1 expression in HAEC (+79% and +140%; n=4; $p<0.05$ vs. Control; figure 6C). Treatment with PIK 75 abrogated basal and TNF-α induced TF (n=4; $p<0.01$ vs. control and; $p<0.01$ versus TNF-α alone;

figure 6A and 6B) and PAI-1 expression (n=4; $p<0.01$ versus TNF-α alone; figure 6D) in HAEC.

Discussion

The small molecule inhibitor PIK 75 is an imidazopyridine developed in the context of a PI3-kinase drug discovery program (12). PIK 75 selectively inhibits the catalytic subunit p110α (IC_{50}= 5.8 nM) encoded by the *PIK3CA* gene (16). Recent studies demonstrate a critical role for p110α in growth factor dependent signaling which may explain the selective mutation and overexpression of p110α associated with several types of cancer. However, the role of p110α activity in regulating vascular smooth muscle and endothelial cell proliferation and migration, two key mechanisms involved in restenosis and reendothelialization following DES implantation, remains unknown.

PDGF-induced VSMC proliferation and migration are crucial cellular mechanisms triggering restenosis in coronary arteries following PTCA and in venous bypass grafts (17). This study demonstrates that pharmacological and genetic inhibition of p110α prevents arterial as well as venous VSMC proliferation *in vitro*. Consistent with this finding, p110α inhibition also impairs smooth muscle cell migration. The PI3K family consists of several isoforms with respect to the regulatory p85 and catalytic p110 subunits. The p85α and p110α isoforms are the most abundant in the vasculature. Studies on the p85α/p110α dimer showed that the p85α regulatory subunit inhibits the catalytic activity of p110α and that oncogenic p85α mutations act by disrupting the inhibitory contact with p110α (18,19). A more recent study demonstrated that phosphorylation of p85α at the serine 83 (pSer83) resulted in inhibition of VSMC and reduced neointima formation after balloon injury (20*).* In line with these findings, the present study demonstrates for the first time that direct pharmacological and genetic inhibition of p110α results in suppression of vascular

SMC proliferation. Since p85α can interact with different p110 isoforms (α,β, and δ) and the expression of the p110 isoforms varies greatly between tissues, modulation of p85α activity and consecutive regulation of downstream cellular responses is likely complex and currently barely understood. In addition, to our knowledge there is currently no pharmacological activator of p85α available and reduction in neointima formation in response to Ser83-mutated p85α is inferior compared with the inhibition in response to rapamycin. In contrast, PIK 75 is a specific small molecule inhibitor of p110α and at the concentrations used in this study (0.1-10 nM) interactions with other isoforms can be excluded (16). Moreover, PIK 75 exerted similar antiproliferative and antimigratory effects on VSMC as compared to rapamycin and paclitaxel suggesting comparable effects on neonintima formation between these compounds, which is of relevance since maximal inhibition of luminal narrowing is a prerequisite in PTCA.

Delayed arterial healing manifested by impaired reendothelialization and enhanced thrombogenicity remains a major concern associated with the use of DES, requiring continuous antiplatelet therapy in patients receiving these stents. Morphological studies have demonstrated a clear delay of reendothelialization in both rapamycin and paclitaxel eluting stents when compared with BMS (21). Indeed, rapamycin and paclitaxel have been shown to suppress EC proliferation and migration in several studies (14,22). In addition, by inducing a senescent phenotype and decrease in eNOS expression, recent data suggest that this drugs not only impair endothelial regrowth but also directly affect endothelial function (23). In line with this observation, rapamycin as well as paclitaxel exert direct prothrombotic effects on EC by enhancing both basal and cytokine induced expression of TF and PAI-1 (24,25). Importantly, most of these biological effects have also been observed with compounds used for second generation DES and suggest that the thrombotic risk of DES is a function of both, impaired endothelial coverage and function (26,27).

In contrast to rapamycin or paclitaxel, inhibition of p110α, with PIK 75 or the corresponding siRNA, did neither affect proliferation nor migration in arterial or venous EC. These findings are supported by previous results showing that cell proliferation occurred normally in EC overexpressing activated p85α (20). Treatment with PIK 75 did neither induce endothelial senescence nor affect eNOS expression, which are both implicated in the endothelial dysfunction induced by rapamycin and paclitaxel. The suppression of basal and cytokine induced TF and PAI-1 expression associated with p110α inhibition is of particular relevance, since both proteins are critically involved in the pathogenesis of stent thrombosis (8). During acute coronary syndromes and PTCA, periprocedural cytokine plasma levels are increased to concentrations high enough to induced TF and PAI-1 expression in surrounding EC (28,29). The present study demonstrates that while rapamycin and paclitaxel enhance both basal and TNF-α induced TF and PAI-1 expression, p110α inhibition with PIK 75 exerts potent antithrombotic effects.

In summary, the present study provides evidence that inhibition of PI3K/p110α differentially regulates SMC and EC. In addition to its potent antiproliferative and antimigratory effects on SMC, targeting p110α also inhibits the expression of prothrombotic markers on EC. Since specific targeting of p110 isoforms is currently evaluated in different areas of modern medicine, we propose local p110α inhibition as a novel strategy in the context of DES design, in order to prevent restenosis without adding the risk of impaired reendeothelialization and enhanced thrombogenicity.

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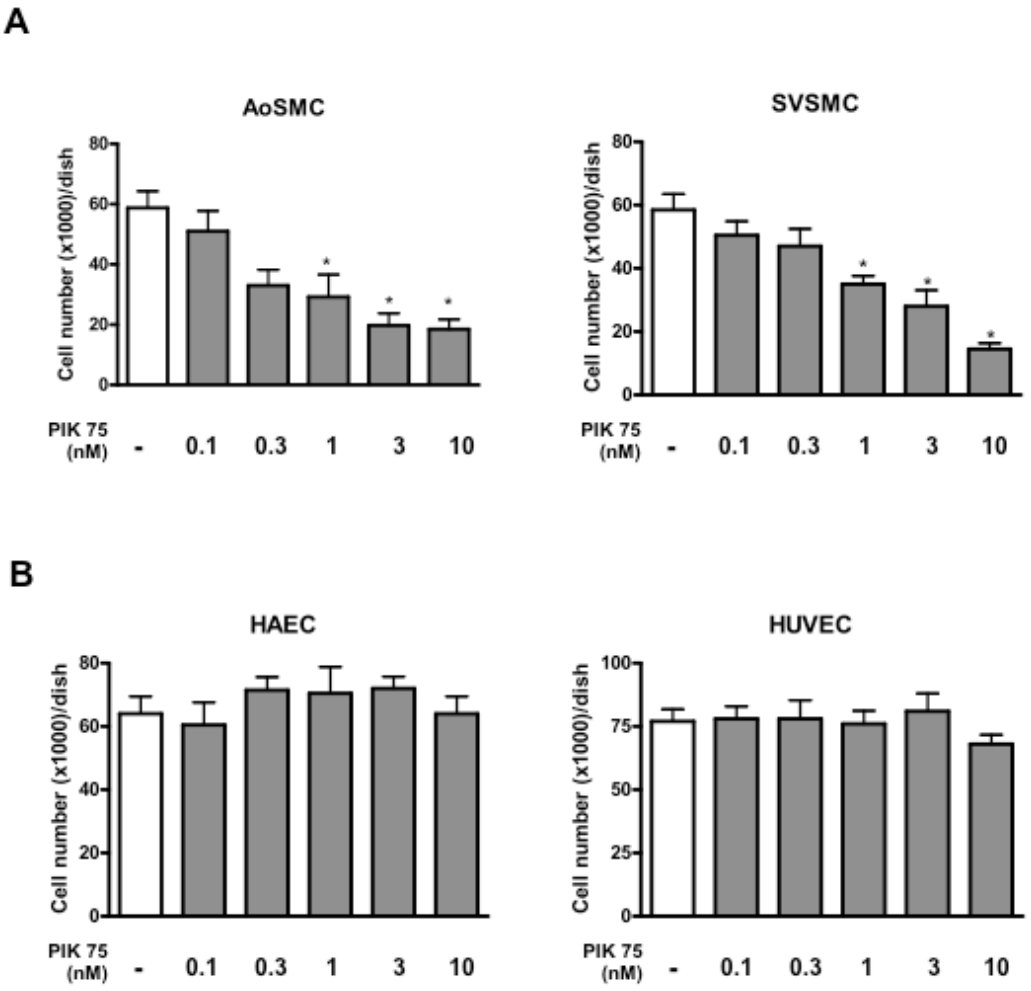
A
AoSMC
SVSMC
Cell number (x1000)/dish
PIK 75 (nM)
-
0.1
0.3
1
3
10
B
HAEC
HUVEC

Figure 1

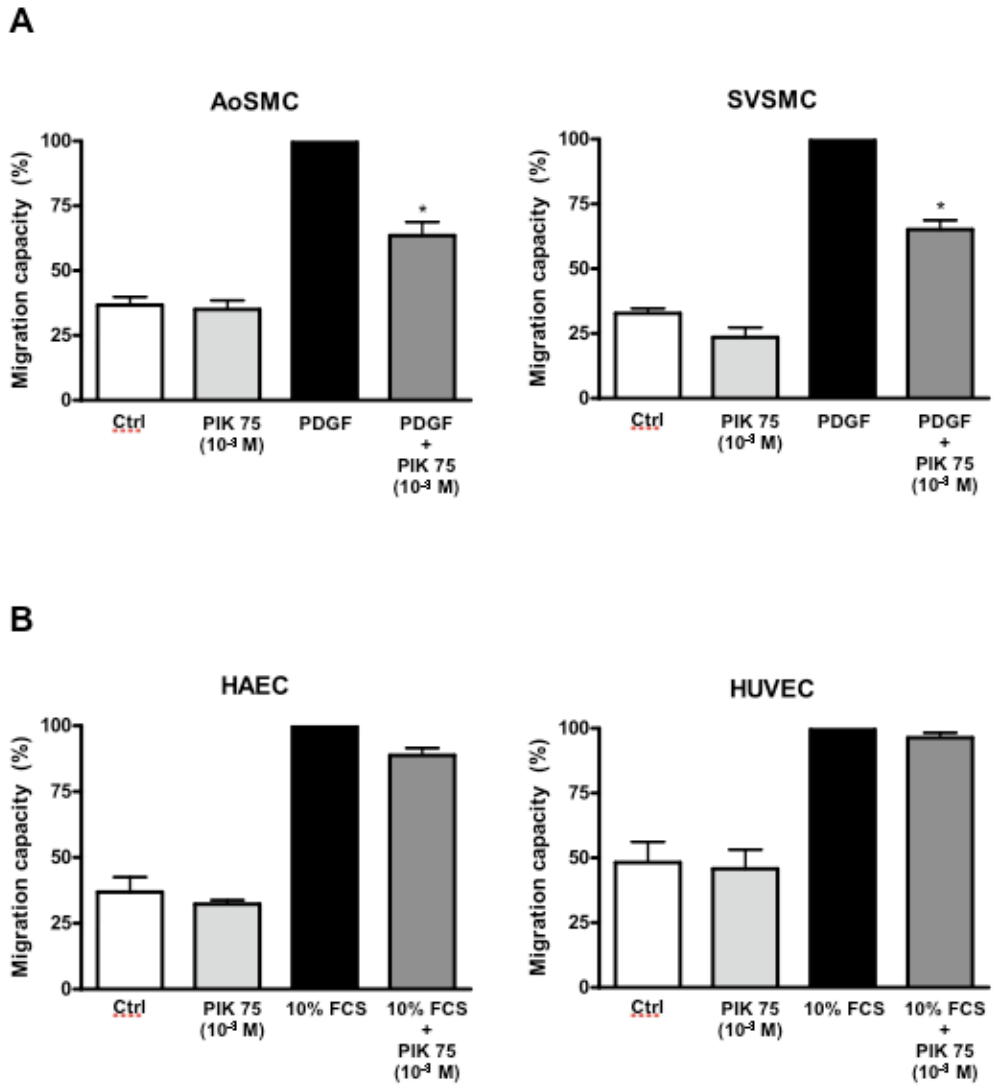
A
AoSMC
SVSMC
Migration capacity (%)
100
75
50
25
0
*
Ctrl
PIK 75 (10-3 M)
PDGF
PDGF + PIK 75 (10-3 M)
B
HAEC
HUVEC
10% FCS
10% FCS + PIK 75 (10-3 M)

Figure 2

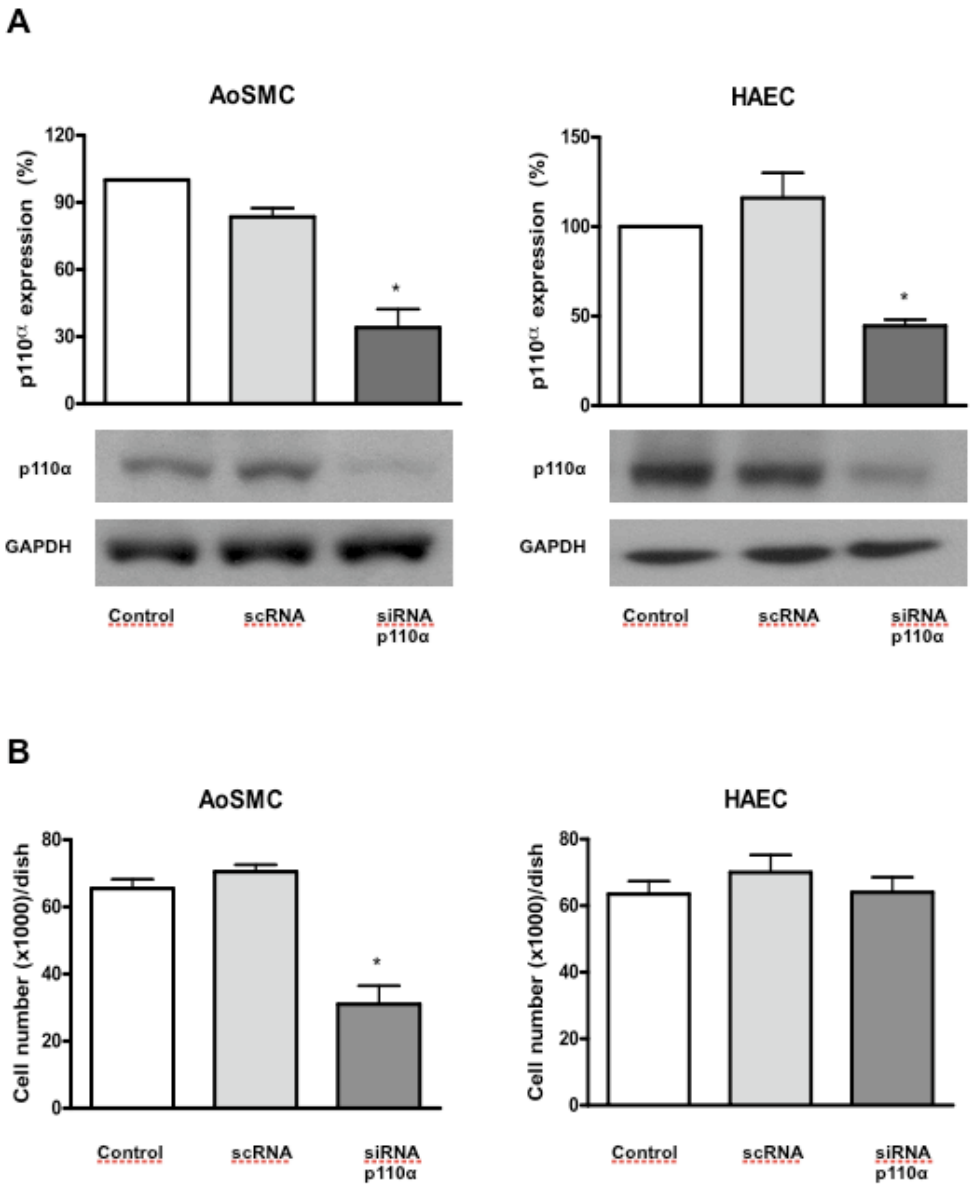
A
AoSMC
HAEC
p110α expression (%)
120
90
60
30
0
150
100
50
*
p110α
GAPDH
Control
scRNA
siRNA p110α
B
Cell number (x1000)/dish
80
40
20

Figure 3

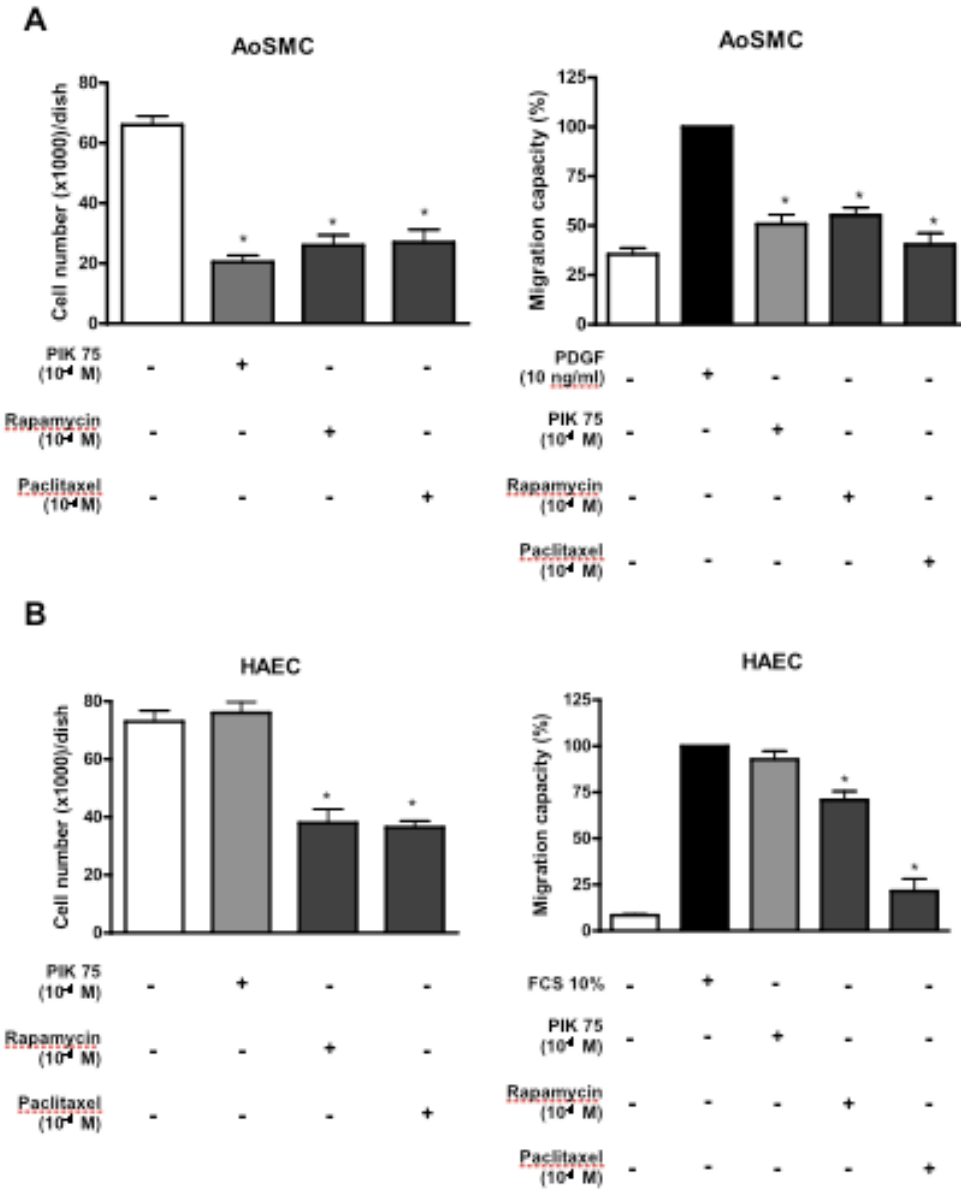
A
AoSMC
Cell number (x1000)/dish
0
20
40
60
80
PIK 75 (10⁻⁴ M) - + - -
Rapamycin (10⁻⁴ M) - - + -
Paclitaxel (10⁻⁴ M) - - - +
AoSMC
Migration capacity (%)
0
25
50
75
100
125
PDGF (10 ng/ml) - + - - -
PIK 75 (10⁻⁴ M) - - + - -
Rapamycin (10⁻⁴ M) - - - + -
Paclitaxel (10⁻⁴ M) - - - - +
B
HAEC
Cell number (x1000)/dish
0
20
40
60
80
PIK 75 (10⁻⁴ M) - + - -
Rapamycin (10⁻⁴ M) - - + -
Paclitaxel (10⁻⁴ M) - - - +
HAEC
Migration capacity (%)
0
25
50
75
100
125
FCS 10% - + - - -
PIK 75 (10⁻⁴ M) - - + - -
Rapamycin (10⁻⁴ M) - - - + -
Paclitaxel (10⁻⁴ M) - - - - +

Figure 4

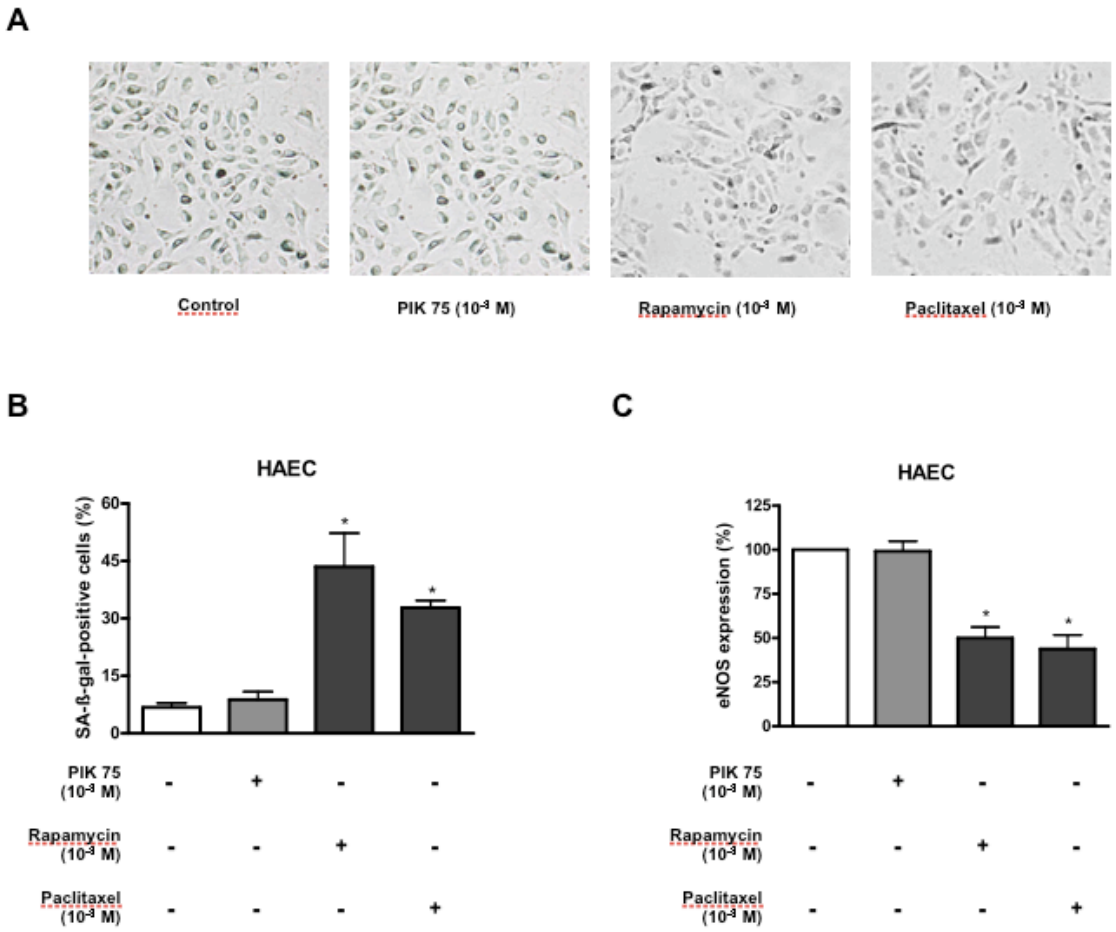
A
Control
PIK 75 (10-3 M)
Rapamycin (10-3 M)
Paclitaxel (10-3 M)
B
HAEC
SA-ß-gal-positive cells (%)
60
45
30
15
0
*
*
PIK 75 (10-3 M) - + - -
Rapamycin (10-3 M) - - + -
Paclitaxel (10-3 M) - - - +
C
HAEC
eNOS expression (%)
125
100
75
50
25
0
*
*
PIK 75 (10-3 M) - + - -
Rapamycin (10-3 M) - - + -
Paclitaxel (10-3 M) - - - +

Figure 5

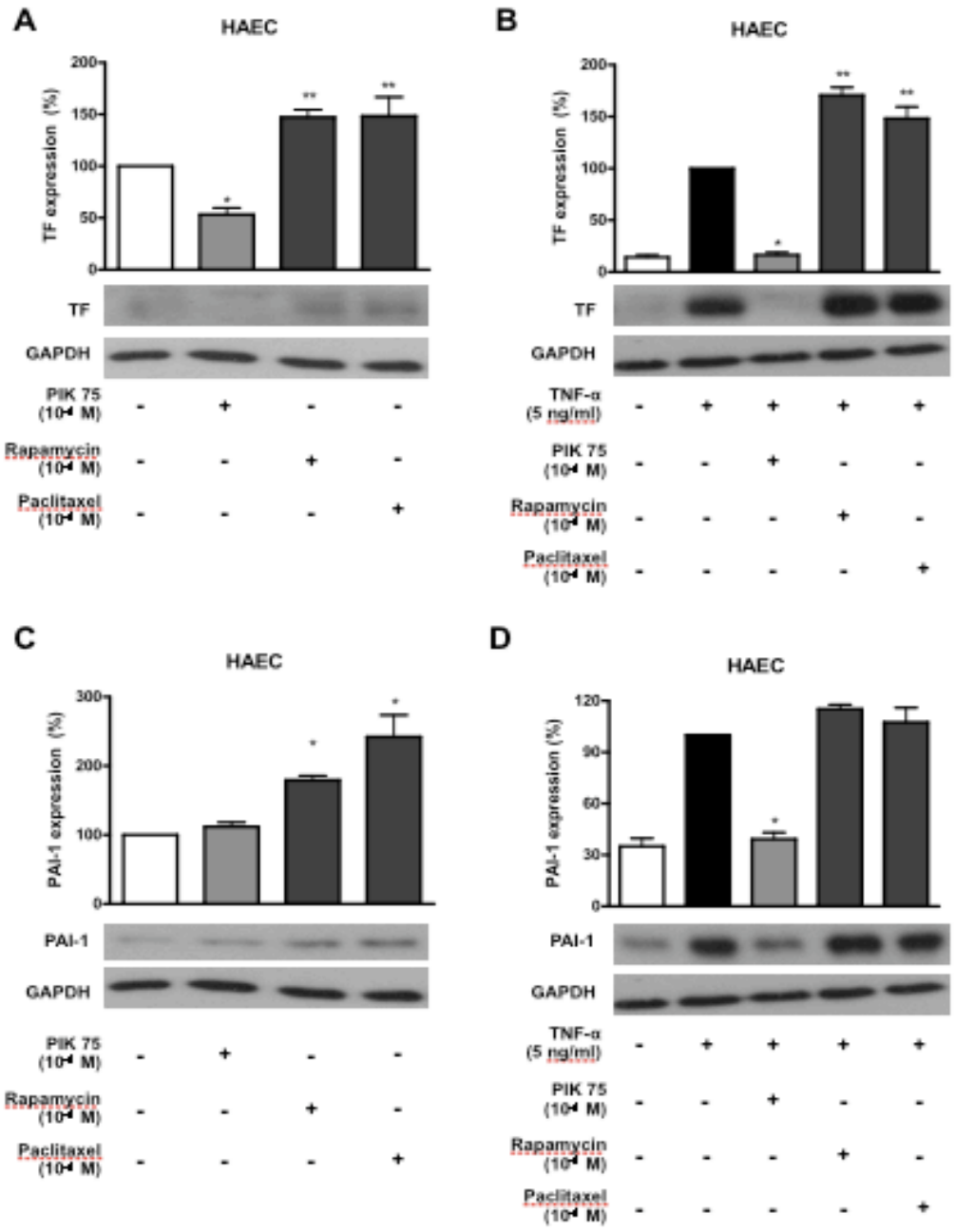
A
HAEC
TF expression (%)
200
150
100
50
0
**
**
*
TF
GAPDH
PIK 75 (10-4 M)
Rapamycin (10-4 M)
Paclitaxel (10-4 M)
B
HAEC
TF expression (%)
TF
GAPDH
TNF-α (5 ng/ml)
PIK 75 (10-4 M)
Rapamycin (10-4 M)
Paclitaxel (10-4 M)
C
HAEC
PAI-1 expression (%)
300
200
100
0
PAI-1
GAPDH
PIK 75 (10-4 M)
Rapamycin (10-4 M)
Paclitaxel (10-4 M)
D
HAEC
PAI-1 expression (%)
120
90
60
30
0
PAI-1
GAPDH
TNF-α (5 ng/ml)
PIK 75 (10-4 M)
Rapamycin (10-4 M)
Paclitaxel (10-4 M)

Figure 6

Figure Legend

Figure 1. PIK 75 inhibits SMC proliferation while sparing endothelial growth.

Treatment with PIK 75 inhibited proliferation of AoSMC and SVSMC in a concentration dependent manner (**A**; *p<0.05 versus control), while proliferation of HAEC and HUVEC was not affected (**B**, p=NS versus control).

Figure 2. PIK 75 prevents SMC but not EC migration.

Treatment with PIK 75 prevented PDGF-BB induced chemotaxis of AoSMC and SVSMC (**A**, *p<0.01 versus control). In contrast, PIK 75 treatment did not affect serum-induced migration of HAEC or HUVEC (**B**, p=NS versus control).

Figure 3. Silencing of p110α inhibits proliferation of AoSMC but not HAEC.

Treatment with the corresponding siRNA targeting resulted p110α resulted in a significant decrease in p110α protein expression in both cell types (**A**, *p<0.01 versus control). Silencing of p110α inhibited proliferation of AoSMC only, while proliferation of HAEC remained unaltered (**B**, p=NS versus control).

Figure 4. Similar anti-proliferative and anti-migratory effects of PIK 75 in AoSMC than rapamycin and paclitaxel.

PIK 75 exerts similar effects on AoSMC migration and proliferation when compared with rapamycin and paclitaxel (**A**, *p<0.005 versus control). In contrast to rapamycin and paclitaxel, treatment with PIK 75 does not affect proliferative and migratory capacity of HAEC (**B**, *p<0.005 versus control).

Figure 5. PIK 75 has no effect on endothelial senescence or eNOS expression.

In contrast to rapamycin or paclitaxel, treatment with PIK 75 does not induce senescence in HAEC as determined by morphological analysis (A, magnification 20x) or β-galactosidase staining (**B**, *p<0.05 versus control and PIK 75 treated cells). Treatment with PIK 75 does not modulate eNOS expression (**C**).

Figure 6. p110α inhibition suppresses endothelial TF and PAI-1 expression.

Treatment with PIK 75 inhibited basal (**A**) and TNF-α induced (**B**) TF expression in HAEC (*p<0.01 vs control and *p<0.01 versus TNF-α alone), whereas rapamycin and paclitaxel enhanced TF expression under both conditions (**p<0.05 versus control and TNF-α alone). Rapamycin and paclitaxel also enhanced PAI-1 expression under basal conditions (**C**, *p<0.05 versus control). Treatment with PIK 75 inhibited TNF-α induced PAI-1 expression in HAEC (**D**, *p<0.01 versus TNF-α alone).

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VIII. Curriculum vitae

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- Assistent, Physiologie Praktikum (Herz-Kreislauf) für Mediziner, HS 2008, Universität Zürich
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- Leistungsstipendium der Medizinischen Universität Wien zur Anerkennung hervorragender Studienleistungen 2004/2005

Forschungsförderungen

- *Targeting vascular PI3K/p110α expression: a new concept in drug eluting stent design?*, Schweizerische Herzstiftung 2011, Hauptantragsteller, *CHF: 82'000*
- *Aging: trigger of arterial thrombosis?*, Schweizerische Herzstiftung 2008, Co-Antragsteller, *CHF: 65'000*
- *Alpha-lipoic acid and arterial thrombus formation*, Novartis Stiftung für medizinisch-biologische Forschung 2007, Co-Antragsteller, *CHF: 55'800*

Gutachter für wissenschaftliche Zeitschriften:

- European Heart Journal
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Zürich, am 14. Dezember 2011